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(54) Title: SPERM FACTOR OSCILLOGENIN

(57) Abstract: The specification describes a novel compound, oscillogenin, which is an active agent in furthering oocyte fertilization by sperm or in parthenogenetic activation of an oocyte. The specification discloses methods of isolating oscillogenin to modulate fertility and to enhance parthenogenetic activation of oocytes for nuclear transfer or in ICSI procedures, and methods of using oscillogenin to test amounts of it in sperm and thus sperm fertility.

# SPERM FACTOR OSCILLOGENIN

## FEDERAL FUNDING

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## CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. provisional application Serial No. 60/191,089 filed on March 22, 2000, which is hereby incorporated in its entirety by reference.

## FIELD OF THE INVENTION

This invention relates to compositions and methods for parthenogenetic activation of oocytes, modulating sperm fertility and assessing sperm fertility. One composition comprises a sperm protein, oscillogenin.

## BACKGROUND OF THE INVENTION

The fields of animal husbandry and artificial reproductive technology (ART) need improved nuclear transfer techniques to increase the efficiency and success rates of the current methods being used. The benefits obtained from artificial reproductive techniques are numerous. For example, the cloning of embryonic cells, together with the ability to transplant the cloned embryonic cells, allows production of several genetically identical animals. Cloning by nuclear transfer is preferable to other methods (*e.g.*, embryo splitting or embryonic cell aggregation to produce fetal placental chimeras), because it allows for (1) the production of multiple copies of genetically identical animals; (2) the selection of specific traits; and (3) the cryogenic storage of the embryonic cells until completion of testing.

### I. Nuclear Transfer

The first successful transfer of a nucleus from an adult mammary gland cell into an enucleated oocyte was reported in 1996 (Campbell *et al.*, *Nature* 380: 64-6 (1996)). Nuclear transfer (NT) involves preparing a cytoplast as a recipient cell. In most cases, the cytoplast is derived from a mature metaphase II oocyte, from which the chromosomes have been removed. A donor cell nucleus is then placed between the zona and the cytoplast. Fusion and cytoplast activation are initiated by electrical stimulation. Successful reprogramming of the donor cell nucleus by the cytoplast is critical, and is a step which may be influenced by cell cycle (Wolf *et al.*, *Biol. Reprod.* 60: 199-204 (1999)).

A number of pregnancies have been established using fetal cells as the source of donor nuclei. However, animal cloning is facilitated by the use of cell lines to create transgenic animals, which allow for the genetic manipulation of the cells *in vitro* before nuclear transfer. *Id.* The mechanisms regulating early embryonic development may be conserved among mammalian species, such that, for example, a bovine oocyte cytoplasm can support the introduced, differentiated, donor nucleus regardless of chromosome number, species or age of the donor fibroblast (Dominko *et al.*, *Biol. Reprod.* 60: 1496-1502 (1999)).

Actively dividing fetal fibroblasts can be used as nuclear donors according to the procedure described in Cibelli *et al.*, *Science* 280: 1256-9 (1998). Additional methods of preparing recipient oocytes for nuclear transfer of donor differentiated nuclei can be performed as described in International PCT Application Nos. 99/05266; 99/01164; 99/01163; 98/3916; 98/30683; 97/41209; 97/07668; 97/07669; and U.S. Patent No. 5,843,754. Typically the transplanted nuclei are obtained from cultured embryonic stem (ES) cells, embryonic germ (EG) cells or other embryonic cells (See, *e.g.*, International PCT Applications Nos. 95/17500 and 95/10599; Canadian Patent No. 2,092,258; Great Britain Patent No. 2,265,909; and U.S. Patent Nos. 5,453,366; 5,057,420; 4,994,384; and 4,664,097). Inner cell mass (ICM) cells can also be used as nuclear donors (Sims *et al.*, *Proc. Natl Acad. Sci. USA* 90: 6143-7 (1990); and Keefer *et al.*, *Biol. Reprod.* 50: 935-9 (1994).

## II. Calcium Induction in Oocytes and Oocyte Activation

Fertilization in mammalian species as well as in other animals is characterized by the presence of calcium ion ($Ca^{2+}$) oscillations, which can last for several hours in mammals (Miyazaki *et al.*, *Dev. Biol.* 118: 259-67 (1986); Wu *et al.*, *Dev. Biol.* 203: 369-81 (1998)); Swann *et al.*, *J. Exp. Zool.* 285: 267-75 (1999). Such $Ca^{2+}$ oscillations are necessary to trigger egg activation and initiate embryonic development (*Id.*), which consists of a sequence of events including cortical granule exocytosis, resumption of meiosis and extrusion of the second polar body, pronuclear formation, DNA synthesis and first mitotic cleavage (Kline *et al.*, *Dev. Biol.* 149: 80-89 (1992); and Schultz *et al.*, *Curr. Topics Dev. Biol.* 30: 21-62 (1995)). The mechanisms by which the sperm initiates $Ca^{2+}$ release are unknown (*Id.*), but three theories are proposed (Swann *et al.*, 1999). First, the sperm acts as a conduit for $Ca^{2+}$ entry into the egg after membrane fusion. Second, the sperm acts on plasma membrane receptors to stimulate a phospholipase C (PLC) within the egg to generate inositol 1,4,5-triphosphate ($InsP_3$ or $IP_3$). Lastly, a sperm may induce $Ca^{2+}$ release by a yet unidentified sperm protein. All but the last have been shown not to be primarily responsible for oocyte activation (Wu *et al.*, *Dev. Biol.* 203: 369-81 (1998)). $IP_3$ mediates $Ca^{2+}$ release by interacting with $IP_3$ receptors ($IP_3R$), which are localized in the endoplasmic reticulum and form tetrameric complexes (Patel *et al.*, *Cell Calcium* 25: 247-64 (1999)). Injection of GTPγ[S], a non-hydrolyzable activator of G-proteins and consequently of PLC, induced repetitive $Ca^{2+}$ responses in eggs of several species, demonstrating that this pathway is functional in mammalian eggs (Miyazaki, *J. Cell. Biol.* 106: 345-53 (1988); and Fissore et al., *Biol. Reprod.* 53: 766-74 (1995)). Furthermore, injection of $IP_3$ has also been shown to induce $Ca^{2+}$ release in mammalian eggs (Miyazaki *et al.*, 1988; Schultz *et al.*, 1995).

There are three defined isoforms of the $IP_3R$ expressed in mammalian eggs (Fissore *et al.*, *Biol. Reprod.* 60: 49-57 (1999); and He *et al.*, *Biol. Reprod.* 61: 935-43 (1999)), although $IP_3R$ subtype 1 ($IP_3R$-1) is expressed abundantly and in overwhelmingly larger amounts than the other isoforms (Parrington *et al.*, *Dev. Biol.* 203: 451-61 (1998); and He *et al.*, *Biol. Reprod.* 57: 1245-55 (1997)). Also, the $IP_3R$-1 protein is expressed in mammalian eggs in a stage-specific manner, suggesting an important role in fertilization. For instance, less than 20 mouse and bovine eggs are required to detect the $IP_3R$-1 protein by Western blotting (He *et al.*, 1997; Fissore *et al.*,

1999), and the amounts of $IP_3R$-1 protein increase significantly during oocyte maturation (Mehlmann *et al., Dev. Biol.* 180: 489-98 (1996); and He *et al.*, 1997). This increase in receptor density results in an increased $IP_3R$ responsiveness during oocyte maturation (Fujiwara *et al., Dev. Biol.* 156: 69-79 (1993); and Mehlmann *et al., Biol. Reprod.* 51: 1088-98 (1994)). Furthermore, injection of the blocking $IP_3R$-1 monoclonal antibody 18A10 prior to insemination inhibited, in a dose-dependent manner, fertilization-associated $Ca^{2+}$ release and activation in mouse eggs (Miyazaki *et al., Science* 257: 251-5 (1992); and Xu *et al., Development* 120: 1851-9 (1994)).

$Ca^{2+}$ release through the $IP_3R$ system may be controlled, in addition to several other mechanisms by regulating the levels of the $IP_3R$-1 protein. Studies in somatic cell lines have shown that $IP_3R$ down-regulation follows persistent stimulation of $IP_3$ production induced by activation of cell surface receptors coupled to PLC (Wojcikiewicz *et al., J. Biol. Chem* 269: 7963-9 (1994); Wojcikiewicz *et al., J. Biol. Chem.* 270: 11678-83 (1995); and Sipma *et al., Cell Calcium* 23: 11-21 (1998)). This degradation of $IP_3R$, which led to decreased cellular responsiveness to $IP_3$, was shown to be specific since it was not accompanied by general protein degradation (Wojcikiewicz *et al., J. Biol. Chem.* 271: 16652-5 (1996); and Bokkala *et al., J. Biol. Chem.* 272: 12454-61 (1997)), was associated with $IP_3$-binding to the $IP_3R$ (Zhu *et al., J. Biol. Chem.* 274: 3476-84 (1999)), and was mediated by the proteasome, a multi-protein cellular complex involved with degradation of ubiquinated proteins (Bokkala *et al.*, 1997; Oberdorf *et al., Biochem. J.* 339: 453-61 (1999)). During fertilization, mammalian eggs also exhibit decreased $IP_3R$ responsiveness as they progress to the pronuclear stage (Fissore *et al., Dev. Biol.* 166: 634-42 (1994); Jones *et al., Development* 121: 3259-66 (1995); and Machaty *et al., Biol. Reprod.* 56: 921-30 (1997)) and this appears to be accompanied by $IP_3R$-1 down-regulation (Parrington *et al.*, 1998; and He *et al., Biol. Reprod.* 61: 935-43 (1999)). However, the mechanism(s) that controls the demise of $IP_3R$-1 in mammalian eggs is not known. Moreover, parthenogenetic activation of mammalian eggs, the use of which has become widespread with the advent of cloning techniques, can be induced by several agonists that stimulate single or multiple $Ca^{2+}$ rises, but their effects on $IP_3R$-1 numbers have not been determined. Thus, we investigated the signaling mechanism that controls

$IP_3R$-1 down-regulation in mouse eggs including the possible involvement of the proteasome pathway.

Sperm cytosolic factors are necessary for oocyte activation (Stice *et al.*, *Mol. Reprod. Dev.* 25: 272-80 (1990) and Swann *et al.*, *Devel.* 110: 1295-302 (1990)). Activation of mammalian oocytes involves exit from meiosis and entry into the mitotic cell cycle by the secondary oocyte, and the formation and migration of pronuclei within the cell. Thus, oocyte activation requires cell cycle transitions. Although fertilization (U.S. Patent No. 5,496,720) and a sperm's cytoplasmic fraction (Swann *et al.*, 1990) can induce $Ca^{2+}$ oscillations, activation can also be induced by parthenogenic treatments that induce single or multiple $Ca^{2+}$ oscillations. Parthenogenetic activation may be used to prepare the oocytes for nuclear transfer.

Parthenogenesis is the production of embryonic cells, with or without eventual development into an adult, from a female gamete in the absence of any contribution from a male gamete (U.S. Patent No. 5,496,720). Parthenogenetic activation of mammalian oocytes can be performed by (1) use of electric shock, electroporation or electrical stimulation; (2) combined treatment with ionomycin and 6-dimethylaminopurine (DMAP); (3) combined treatment with the calcium ionophore A23187 and 6-DMAP (Susko-Parrish *et al.*, *Dev. Biol.* 166: 729-39 (1994); Mitalipov *et al.*, *Biol. Reprod.* 60: 821-7 (1999); Liu *et al.*, *Biol Reprod.* 61: 1-7 (1999); and U.S. Patent No. 5,496,720). The latter two methods use calcium ionophores in combination with protein kinase inhibitors, which are important for inducing protein kinase inhibitor release (Mayes *et al.*, *Biol. Reprod.* 53: 270-5 (1995)).

Other divalent cations utilized for oocyte activation include magnesium, strontium, barium or calcium, *e.g.*, in the form of an ionophore. Divalent cation levels can also be increased by means of electric shock, oocyte treatment with ethanol, and treatment with caged chelators. Phosphorylation in oocytes may be reduced by addition of kinase inhibitors (*e.g.*, serine-threonine kinase inhibitors, such as 6-dimethylaminopurine, staurosporine and sphingosine) (U.S. Patent No. 5,945,577). Alternatively, oocyte protein phosphorylation may be inhibited by introducing a phosphatases into the oocyte (*e.g.*, phosphatase 2A and phosphatase 2B) (*Id.*).

Alternatively, activation may be achieved by application of known activation agents. For example, penetration of oocytes by sperm during fertilization has been shown to yield greater numbers of viable pregnancies and multiple genetically identical calves after nuclear transfer. Also, treatment with electrical and chemical shock may be used to activate NT embryos after fusion. Suitable oocyte activation methods are the subject of U.S. Patent No. 5,496,720, to Susko-Parrish *et al.*, herein incorporated by reference in its entirety.

***Oscillin.*** Several groups postulated that sperm activates oocytes via a protein which induces $Ca^{2+}$ oscillation. The putative proteins were termed oscillogen (Parrington *et al.*, *Nature* 379: 364-8 (1996)). Oscillin was the first identified oscillogens, and was believed to induce intracellular calcium release in oocytes (*Id.*). Oscillin in fact is glucosamine 6-phosphate deaminase (Wolosker *et al.*, *FASEB J.* 12: 91-9 (1998)). However, despite experiments that purportedly demonstrated that oscillogen induced oocyte activation to the same extent as oocyte injection with a spermatid nuclei (Sasagawa *et al.*, *J. Urol.* 158: 2006-8 (1997); Wolny *et al.*, *Mol. Reprod. & Dev.* 52: 277-87 (1999)), oscillin was later demonstrated not to be the sperm protein responsible for $Ca^{2+}$ release in oocytes (Wolosker *et al.*, (1998); and Wu *et al.*, *Dev. Biol.* 203: 369-81 (1998)). As a consequence, the sperm factor responsible for oocyte activation remains unknown (Wolny *et al.*, 1999).

## III. Preparing Somatic Cells for Nuclear Transplantation or Nuclear Transfer

For purposes of animal husbandry, nuclear transfer can be used with embryonic stem cells (ES), inner cell mass cells (ICMs) and somatic cells.

***Embryonic Stem Cells.*** Another system for producing transgenic animals has been developed that uses ES cells. In mice, ES cells have enabled researchers to select for transgenic cells and perform gene targeting. This method allows more genetic engineering than is possible with other transgenic techniques. For example, ES cells are relative-ly easy to grow as colonies *in vitro*, can be transfected by standard procedures, and the transgenic cells clonally selected by antibiotic resistance (Doetschman, "Gene transfer in embryonic stem cells." IN TRANSGENIC ANIMAL TECHNOLOGY: A LABORATORY HANDBOOK 115-146 (C. Pinkert, ed., Academic Press, Inc., New York

1994)). Furthermore, the efficiency of this process is such that sufficient trans-genic colonies (hundreds to thousands) can be produced to allow a second selection for homologous recombinants (*Id.*). ES cells can then be combined with a normal host embryo and, because they retain their potency, can develop into all the tissues in the resulting chimeric animal, including the germ cells. Thus, the transgenic modification is transmissible to subsequent generations.

Methods for deriving embryonic stem (ES) cell lines *in vitro* from early preimplantation mouse embryos are well known (Evans *et al.*, *Nature* 29: 154-6 (1981); and Martin, *Proc. Natl. Acad. Sci. USA* 78: 7634-8 (1981)). ES cells can be passaged in an undifferentiated state, provided that a feeder layer of fibroblast cells (Evans *et al.*, 1981) or a differentiation inhibiting source (Smith *et al.*, *Dev. Biol.* 121: 1-9 (1987)), is present.

In view of their ability to transfer their genome to the next generation, ES cells have potential utility for germ line manipulation of livestock animals. Some research groups have reported the isolation of pluripotent embryonic cell lines. For example, Notarianni *et al.*, *J. Reprod. Fert. Suppl.* 43: 55-260 (1991) reported the establishment of stable, pluripotent cell lines from pig and sheep blastocysts, which exhibit some morphological and growth characteristics similar to that of cells in primary cultures of inner cell masses (ICMs) isolated immunosurgically from sheep blastocysts. Also, Notarianni *et al.*, *J. Reprod. Fert. Suppl.* 41: 51-56 (1990) disclosed maintenance and differentiation in culture of putative pluripotent embryonic cell lines from pig blastocysts. Gerfen *et al.*, *Anim. Biotech.* 6: 1-14 (1995) disclosed the isolation of embryonic cell lines from porcine blastocysts, which do not require mouse embryonic fibroblast feeder layers and reportedly differentiate into several different cell types during culture.

Further, Saito *et al.*, *Roux's Arch. Dev. Biol.* 201: 134-41 (1992) reported cultured, bovine embryonic stem cell-like cell lines, which survived three passages, but were lost after the fourth passage. Handyside *et al.*, *Roux's Arch. Dev. Biol.* 196: 185-90 (1987) disclosed culturing immunosurgically isolated sheep embryo ICMs under conditions that allow for the isolation of mouse ES cell lines derived from mouse ICMs.

Campbell *et al.*, *Nature* 380: 64-6 (1996) reported the production of live lambs following nuclear transfer of cultured embryonic disc (ED) cells from day nine ovine

embryos cultured under conditions which promote the isolation of ES cell lines in the mouse.

Purportedly, animal stem cells have been isolated, selected and propagated for use in obtaining transgenic animals (see Evans *et al.*, WO 90/03432; Smith *et al.*, WO 94/24274; and Wheeler *et al.*, WO 94/26884). Evans *et al.* also reported the derivation of purportedly pluripotent ES cells from porcine and bovine species, which purportedly are useful for the production of transgenic animals.

ES cells from a transgenic embryo can be used in nuclear transplantation. The use of ungulate ICM cells for nuclear trans-plantation also has been reported. In the case of live-stock animals (*e.g.*, ungulates) nuclei from similar preimplantation livestock embryos support the development of enucleated oocytes to term (Keefer *et al.*, *Biol. Reprod.* 50: 935-39 (1994); Smith *et al.*, *Biol. Reprod.* 40: 1027-1035 (1989)). In contrast, nuclei from mouse embryos do not support development of enucleated oocytes beyond the eight-cell stage after transfer (Cheong *et al.*, *Biol. Reprod.* 48: 958-63 (1993)). Therefore, ES cells from livestock animals are highly desirable, because they may provide a potential source of totipotent donor nuclei, genetically manipulated or other-wise, for nuclear transfer procedures.

***Use of ICM Cells.*** Collas *et al.*, *Mol. Reprod. Dev.* 38: 264-7 (1994) disclosed nuclear transplantation of bovine ICMs by microinjection of the lysed donor cells into enucleated mature oocytes. Culturing of embryos *in vitro* for seven days produced fifteen blastocysts which, upon transfer into bovine recipients, resulted in four pregnancies and two births. Also, Keefer *et al.* (1994) disclosed the use of bovine ICM cells as donor nuclei in nuclear transfer procedures, to produce blastocysts which also resulted in several live offspring. Further, Sims *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 6143-7 (1993) disclosed the production of calves by transfer of nuclei from short-term *in vitro* cultured bovine ICM cells into enucleated mature oocytes.

## IV. Intracytoplasmic Sperm Injection (ICSI)

Sperm can be obtained by one of several methods including microsurgical epidiymal sperm aspiration (MESA) and testicular sperm extraction (TESE). In instances of mature epidiymal spermatozoa and testicular spermatozoa, when injected into mature mouse oocytes, normal embryo development and resulting mice occur (Sasagawa *et al.*, *J. Urol.* 158: 2006-8 (1997)). However, round spermatids are unable to activate oocytes (*Id.*). Therefore, it for purposes of animal husbandry as well as for artificial reproductive techniques, the simultaneous injection of oscillogenin into oocytes can be used to initiate normal embryo development when using immature sperm or round spermatids.

ICSI is a technique developed for use in artificial reproduction and *in vitro* fertilization in the ART field to assist men with defective sperm. Some have suggested that this procedure has revolutionized the treatment of male infertility, as normal fertilization can not be achieved with severely affected spermatozoa (Tarlatzis *et al.*, *Hum. Reprod.* 13S: 165-77 (1998)). For example, cystic fibrosis has been suggested to cause congenital aphasia of the vas deferens, which reduces sperm quality (Jakubiczka *et al.*, *Hum. Reprod.* 14: 1833-4 (1999)). Other causes of male infertility include Y-chromosomal microdeletions leading to spermatogenic impairment and karyotype abnormalities (Kim *et al.*, *Prenat. Diagn.* 18: 1349-65 (1998)). Sperm effectiveness can also be decreased as a result of exposure to protamine (Ahmadi *et al.*, *J. Assist. Reprod. Genet.* 16: 128-32 (1999)). ICSI is also relevant to animal husbandry (see, *e.g.*, Li *et al.*, *Zygote* 7: 233-7 (1999)).

Gomez *et al.*, *Reprod. Fertil. Dev.* 10: 197-205 (1998), suggested that the presence of calcium in the media enhanced fertilization rates after ICSI. This was not unexpected, as Sousa *et al.*, *Mol. Hum. Reprod.* 2: 853-7 (1996), suggested that a soluble sperm factor (SSF) was likely responsible for the $Ca^{2+}$ oscillations driving oocyte activation after ICSI. The $Ca^{2+}$ wave may be large enough to generate all the responses associated with fertilization (Uranga *et al.*, *Int'l. J. Dev. Biol.* 40: 515-9 (1996)). Additionally, the absence of the typical oscillatory $Ca^{2+}$ response in spermatocyte-injected oocytes is presumed to be due to the actual deficiency of SSF in the spermatocytes, rather than to defective responsiveness of the injected oocytes or to the failure of SSF release

into the oocyte cytoplasm (Sousa *et al.* (1996)). Additionally, the calcium response may be important for normal embryonic development after spermatid conception (*Id.*). Tesarik *et al.*, *Biol. Reprod.* 51: 385-91 (1994) reported that although $Ca^{2+}$ oscillations are observed in ICSI fertilized oocytes, it occurs only after a considerable delay.

ICSI is also used in techniques to karyotype human spermatozoa with poor fertilization capacity of sperm. For, example, Goud *et al.*, *Hum. Reprod.* 13: 1336-45 (1998) discusses techniques and conditions for assessing parthenogenetic activation of Syrian golden hamster oocytes microinjected with human spermatozoa.

Sperm fertility also can be assessed using antibodies directed to heparin binding proteins and assessing the heparin binding protein content of sperm membranes (U.S. Patent No. 5,962,241). Using the sperm factor, oscillogenin, described herein, may help to overcome certain growth abnormalities which may result from the manipulation of preimplantation embryos *in vitro* (*e.g.*, large calf syndrome) caused perhaps by genetic imprinting (Moore *et al.*, *Rev. Reprod.* 1: 73-7 (1996)) or perhaps reducing the high lethality of reconstructed embryos. Genetic imprinting is related to protein kinase activity, which in turn may be controlled, in part by the calcium ion oscillations observed upon normal sperm-induced fertilization of a mature oocyte. Such developmental abnormalities can be detrimental and even lethal to the afflicted animal. Additional methods of determining sperm fertility are discussed in U.S. Patent Nos. 5,770,363; 5,763,206; 5,434,057; and 5,358,847.

Therefore, notwithstanding what has previously been reported in the literature, there exists a need for im-proved methods of inducing oocytes $Ca^{2+}$ oscillations for activating oocytes, especially for use with nuclear transfer and ICSI and other related artificial reproductive technologies. Additionally, methods of making and using oscillogenin and agents regulating oscillogenin will greatly aid the production of cloned livestock, the use of ART for the birth of healthy humans, and for contraception.

## OBJECTS AND SUMMARY OF THE INVENTION

It is an object of the invention to provide a novel method of isolating oscillogenin from sperm comprising: (A) preparing a sperm cytoplasmic fraction; (B) isolating oscillogenin by sequentially processing the sperm cytoplasmic fraction through a HiTrap blue affinity FPLC chromatographic column, a hydroxyapatite FPLC column, and a surperose 12 FPLC chromatographic column; and (C) obtaining a fraction with $[Ca^{2+}]_i$ releasing activity.

It is another object of the invention to provide a method of enhancing oocyte activation of an oocyte comprising the step of injecting oscillogenin into an oocyte prior to, simultaneously with, or immediately after injecting or fusing the oocyte with a sperm or other cell nuclei, wherein said oocyte has been treated, before or after oscillogenin injection, to remove or inactivate its endogenous nucleus. The oocyte can be a mammalian oocyte (*e.g.*, primate, bovine, caprine, ovine, porcine, feline, murine, or canine), and may be preferably a human oocyte. The method may further comprise the step of injecting the oocyte with at least one agent which additionally enhances divalent cation release or a combination of such agents.

It is a more specific object of the invention to allow the activated oocyte to develop into an embryo, and in some circumstances, this embryo may be implanted into a female surrogate and allowed to gestate into a non-human animal.

It is another object of the invention to provide a method of enhancing intracytoplasmic sperm injection (ICSI) comprising the step of injecting an oocyte with oscillogenin either before or after a sperm or sperm nucleus is inserted into the oocyte. Another objection of the invention is to provide a method of enhancing parthenogenetic activation of an oocyte comprising the step of injecting an oocyte with oscillogenin.

It is another object of the invention to provide a method of predicting sperm $[Ca^{2+}]_i$ releasing activity comprising measuring oscillogenin concentration in a sperm sample. It is a more specific object to also provide a kit for predicting sperm $[Ca^{2+}]_i$ releasing activity comprising a labeled agent which recognizes and binds to oscillogenin or a nucleic acid encoding oscillogenin.

It is a further object of the invention to provide a nucleic acid encoding an oscillogenin, as well as its corresponding amino acid sequence. The oscillogen sequence

can be human, primate, bovine, porcine, ovine, equine, feline, canine, murine and caprine.

Another object of the invention provides an antibody or immunogenic fragment thereof which recognizes and binds to oscillogenin. Preferably the antibody is a monoclonal antibody and the immunogenic fragment is consisting of: Fab, scFv, $F(ab')_2$ and Fab'.

## BRIEF DESCRIPTION OF THE FIGURES

**Figure 1.** Panel A shows the step-wise use of chromatographic columns utilized to obtain elutions enriched in sperm factor (SF) proteins. Sperm fractions are first processed through a HiTrap blue dye column, then followed by a hydroxyapatite column and lastly by a Superose 12 column. Panel B shows the distribution of proteins in the various fractions (F1-F5) separated using polyacrylamide gel electrophoresis after processing through the various chromatographic columns. Panel C shows the $[Ca^{2+}]_i$ releasing activity of each of the fractions from the multi-column. The greatest activity monitored is in the F4-1 fraction.

**Figure 2.** Western blot analysis and quantification of $IP_3R$-1 immunoreactivity in mouse eggs aged *in vitro* (A and B) or following *in vivo* fertilization C and D). Twenty eggs (e) were used in each lane. "UF" stands for unfertilized and "F" for fertilized eggs. MII eggs were always at 16 hr post human chorionic gonadotrophin ("hCG") and the indicated times refer to hr post hCG (phCG). Data are presented as means±SEM. Treatments under bars that share a common superscript are not significantly different ($p > 0.05$). Values are the mean of 4 Western-blotting experiments, performed on different batches of eggs.

**Figure 3.** Western blot analysis and quantification of $IP_3R$-1 immunoreactivity in mouse eggs activated by exposure to 7% ethanol (Et; A and B) or ionomycin/DMAP (Io/D; C and D). Activation was started at 16 hr phCG. Et-24hr-1-cell were those eggs that at 24 hr phCG exhibited pronuclear formation after ethanol exposure; and Et-24hr-2 cell were those that divided into two-cells within 4 hr of exposure. All eggs at 24 hr phCG (8 hr post activation) for all treatments exhibited pronuclear formation. Twenty eggs (e) were used per lane. Treatments under bars with

different superscripts are significantly different ($p < 0.05$). Values are the mean of three Western-blotting experiments, performed on different batches of eggs.

**Figure 4.** Western blot analysis and quantification of $IP_3R$-1 immunoreactivity in mouse eggs activated by injection of SF (A and B) or adenophostin A (Ad; C and D). Injections were carried out at 16 hr phCG and samples taken within 1 hr, 2 hr, 4 hr, and 8 hr post-injection. Treatments under bars with different superscripts are significantly different ($p < 0.05$). Values are the mean of three Western-blotting experiments, performed on different batches of eggs.

**Figure 5.** Western blot analysis and quantification of $IP_3R$-1 immunoreactivity in mouse eggs activated by $SrCl_2$ (A and B) or thimerosal ("Th"; C and D). Activation was carried out at 16 hr phCG and 15 eggs were used per lane. "C" stands for control and these eggs were cultured for the same amount of time but were not exposed to $SrCl_2$. Treatments under bars that do not share common superscripts are significantly different ($p < 0.05$). Values are the mean of five Western-blotting experiments, performed on different batches of eggs.

**Figure 6.** $[Ca^{2+}]_i$ oscillations profiles in mouse eggs induced by several of the agonists used in this study. Injection of SF (~10 ng/μl intracellular concentration) induced highly repetitive rises (A) similar to those induced by injection of adenophostin A (B; ~100 nM intracellular concentration). $SrCl_2$ induced prolonged rises and of slower frequency (C). Thimerosal, which was incubated with eggs for 30 min, induced repetitive rises (D). The presented $Ca^{2+}$ recordings were carried out in three separate experiments, and the termination of the oscillations was due to the termination of the recordings rather than to cessation of the oscillations.

**Figure 7.** Western blot analysis and quantification of $IP_3R$-1 immunoreactivity in mouse eggs activated by SF in the presence or absence of lactacystin ("Lac"). Injection of SF was carried out at 16 hr phCG. Eggs were preincubated with the inhibitor (100 μM) for 30 min. prior to SF injection and were cultured in Lac for 2 hr after the injections. Fifteen eggs (e) were used per lane. Treatments under bars that do not share common superscripts are significantly different ($p < 0.05$). Values are the mean of 3 Western blotting experiments, performed on different batches of eggs.

## DETAILED DESCRIPTION OF THE INVENTION

This invention is directed towards methods of isolating oscillogenin from sperm as well as recombinant production of oscillogenin. The protein can then be used to enhance parthenogenetic activation of oocytes and enhance sperm fertility. Detection of the protein or nucleic acids which encode the protein can be used to assess sperm fertility. The invention also considers modulation of oscillogenin activity to thereby regulate fertility.

### I. Definitions

By "oscillogenin" is meant the protein responsible for $Ca^{2+}$ oscillations when injected into an unactivated oocyte. In the instance of oscillogenin purified from sperm cell extracts, the "purified oscillogenin" is obtained by sequentially processing the oscillogenin through at least three chromatographic columns and obtaining the $[Ca^{2+}]_i$ releasing fractions. Said fractions will comprise oscillogenin and about five other proteins as assessed by silver staining. More preferred purified oscillogenin compositions will comprise oscillogenin and about three other proteins as determined by silver staining. The preferred sequential chromatographic columns used is as described in Example 1. By "sequentially processing" is meant the columns used preferably in the order described in Example 1.

By "nucleic acid" or "nucleic acid molecule" is meant to include a DNA, RNA, mRNA, cDNA, or recombinant DNA or RNA.

By "animal" is meant any member of the animal kingdom including vertebrates (*e.g.*, frogs, salamanders, chickens, or horses) and invertebrates (*e.g.*, worms, *etc.*). Preferred animals are mammals. Preferred mammalian animals include livestock animals (*e.g.*, ungulates, such as bovines, buffalo, equines, ovines, porcines and caprines), as well as rodents (*e.g.*, mice, hamsters, rats and guinea pigs), canines, felines and primates. By "non-human" is meant to include all animals, especially mammals and including primates other than human primates.

By "female surrogate" is meant a female animal into which an embryo of the invention is inserted for gestation. Typically, the female animal is of the same animal

species as the embryo, but the female surrogate may also be of a different animal species. The embryo, as used herein, can include a complex of two or more cells.

By "cytoplast" is meant the fragment of the cell remaining once the nucleus is removed.

By "parthenogenetic activation" is meant development of an ovum or oocyte without fusion of its nucleus with a male nucleus or male cell to form a zygote.

By "oocyte" is meant an animal egg, nucleated or enucleated which has not undergone a $Ca^{2+}$ oscillations.

By "activated oocyte" is meant an oocyte which acts as though it has been parthenogenically activated or as though it has been fertilized.

By "enucleated oocyte" is meant an animal egg which has had its endogenous nucleus removed or inactivated.

By "sperm," "semen," "sperm sample," and "semen sample" are meant the ejaculate from a male animal which contains spermatozoa. A mature sperm cell is a "spermatozoon," whereas the precursor is a "spermatid." Spermatids are the haploid products of the second meiotic division in spermatogenesis, which differentiate into spermatozoa.

By "sperm fertility" is meant the ability of a sperm to fertilize an egg and create an embryo. By "sperm $[Ca^{2+}]_i$-releasing activity" is meant the ability of a sperm to activate an oocyte (of any animal), which can be measured by induction of $Ca^{2+}$ oscillations in the oocyte.

By "sperm cytoplasmic fraction" is meant the portion of the cell which lacks the nucleus and most of the genetic material. Preferably, the cytoplasm fraction comprises the substances contained within the plasma membrane but excluding the nucleus and its genetic material.

By "inducing", "increasing," "enhancing" or "up-regulating" is meant the ability to raise the level of oscillogenin activity. By "enhancing activation" is meant a method or agent which increases oocyte activation.

By "modulating" or "regulating" is meant the ability of an agent to alter (*e.g.*, up-regulate or down-regulate) from the wild type level observed in the individual

organism the activity of oscillogenin. Oscillogenin activity can be at the level of transcription, translation, nucleic acid or protein stability or protein activity.

By "antibody fragment" and "immunogenic fragment"is meant an immunogenic protein peptide capable of recognizing and binding to oscillogenin or a fragment thereof. This includes an anti-oscillogenin antibody or polypeptide fragment thereof.

By "intracytoplasmic sperm injection" or "ICSI" is meant injection of a sperm or at least the genetic contents of a sperm into an oocyte.

The terms "nuclear transfer" or "nuclear transplantation" refer to a method of cloning, wherein the donor cell nucleus is transplanted into a cell before or after removal of its endogenous nucleus. The cytoplast could be from an enucleated oocyte, an enucleated ES cell, an enucleated EG cell, an enucleated embryonic cell or an enucleated somatic cell. Nuclear transfer techniques or nuclear transplantation techniques are known in the literature (Campbell *et al.*, *Theriogenology* 43: 181 (1995); Collas *et al.*, *Mol. Reprod. Dev.* 38: 264-267 (1994); Keefer *et al.*, *Biol. Reprod.* 50: 935-939 (1994); Sims *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 6143-6147 (1993); Evans *et al.*, WO 90/03432; Smith *et al.*, WO 94/24274; and Wheeler *et al.* , WO 94/26884. Also U.S. Patent Nos. 4,994,384 and 5,057,420 describe procedures for bovine nuclear transplantation. In the subject application, "nuclear transfer" or "nuclear transplantation" or "NT" are used interchangeably.

The terms "nuclear transfer unit" and "NT unit" refer to the product of fusion between or injection of a somatic cell or cell nucleus and an enucleated cytoplast (*e.g.*, an enucleated oocyte), which is some-times referred to herein as a fused NT unit.

By "somatic cell" is meant any cell of a multicellular organism, preferably an animal, that does not become a gamete.

By "isolated" or "purified" oscillogenin is meant a $Ca^{2+}$-activity protein substantially purified from either the sperm it is isolated from or the cell used to recombinantly prepare the oscillogenin protein or peptide from other non-oscillogenin proteins, peptides or nucleic acids.

By "protein kinase inhibitor" is an agent which inhibits an enzyme that catalyzes the transfer of phosphate from ATP to hydroxyl side chains on proteins causing changes of function of the protein. The preferred protein kinase inhibitors of this invention are

6-dimethylaminopurine (DMAP), staurosporine, butyrolactone, roscovitine, p34(cdc2) inhibitors, 2-aminopurine and sphingosine.

By "phosphatase" is meant an enzyme that hydrolyzes phosphomonoesters. The preferred phosphatases described herein are phosphatase 2A and 2B.

By "calcium ionophore" are agents which allow calcium ions ($Ca^{+2}$) to cross lipid bilayer. Preferred calcium ionophores include ionomycin and A23187.

By "differentiate" or "differentiation" is meant to refer to the process in development of an organism by which cells become specialized for particular functions. Differentiation requires that there is selective expression of portions of the genome.

By "inner cell mass" or "ICM" is meant a group of cells found in the mammalian blastocyst that give rise to the embryo and are potentially capable of forming all tissues, embryonic and extra-embryonic, except the trophoblast.

By "feeder layer" is meant a layer of cells to condition the medium in order to culture other cells, particularly to culture those cells at low or clonal density.

By "medium" or "media" is meant the nutrient solution in which cells and tissues are grown.

The term "pharmaceutically acceptable carrier" , as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent. The diluent or carrier ingredients should not be such as to diminish the therapeutic effects of the active compound(s).

The term "composition" as used herein means a product which results from the mixing or combining of more than one element or ingredient.

## II. Method of Isolating Oscillogenin from Sperm

Sperm fractions with oscillogenin can be obtained from animal sperm by first preparing cytosolic sperm extracts as described by Wu *et al.*, *Mol. Reprod. Devol.* 46: 176-89 (1997) and Wu *et al.*, *Mol. Reprod. Dev.* 49: 37-47 (1998). Briefly, semen samples are washed twice with TL-Hepes medium, and the sperm pellet is resuspended in a solution containing 75 mM KCl, 20 mM Hepes, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM glycerophosphate, 1 mM DTT, 200 μM PMSF, 10 μg/ml pepstatin,

10 μg/ml leupeptin, at pH 7.0. The sperm suspension is sonicated for about 25 to 35 minutes at 4°C. (XL2020, Heat Systems, Inc., Farmingdale, NY). The lysate is then spun twice at 10,000 x g and, the supernatants collected. The resulting supernatant is then centrifuged at 100,000 x g for one hour at 4°C. This clear supernatant represents the cytosolic fraction of the sperm. Active sperm fractions can also be obtained from, for example, pig sperm by freezing and thawing the sperm twice in the absence of a cryoprotectant.

The cytosolic fraction thus obtained is then subjected ammonium sulfate precipitation (50%) and precipitated. The precipitate can then be pelleted by centrifugation and stored at -20 to -80°C. for prolonged periods of time. The pellet can be reconstituted and subjected to the following chromatographic procedures for isolation and/or purification of oscillogenin. The reconstituted pellet is first subjected to a hydroxyapatite FPLC chromatographic column, followed by a chromatofocusing column, and then followed by a Superose 12 FPLC chromatographic column. The fraction eluting at a molecular weight of approximately 30 to about 68 kDa contains the $[Ca^{2+}]_i$-inducing agent, oscillogenin. The conditions for of the chromatographic columns can be used as described for the individual chromatographic columns used in Wu *et al.*, 1998. Wu *et al.*, (1998) do not describe the specific sequential use of the chromatography columns as described herein, nor do Wu *et al.* describe which specific fractions are to be used that contain the activation factor(s).

## III. Characterization of Oscillogenin

Once the oscillogenin is isolated from sperm, it can be peptide sequenced. Using the peptide sequences thus identified, degenerate probes can be created which can be used to screen libraries to identify the gene, which encodes oscillogenin.

The present invention further provides nucleic acid molecules that encode oscillogenin and related proteins, preferably in isolated form. As used herein, "nucleic acid" is defined as RNA, rRNA, mRNA, DNA, rDNA or cDNA sequences which encode oscillogenin or a polypeptide fragment thereof, or is complementary to nucleic acid sequence encoding oscillogenin or a polypeptide fragment thereof, or hybridizes to such a nucleic acid and remains stably bound to it under appropriate stringency conditions, or

encodes a polypeptide sharing at least 75% sequence identity, or preferably at least 80%, or more preferably at least 85%, or most preferably at least about 90-95% identify with the peptide sequences. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such hybridizing or complementary nucleic acids, however, are defined further as being novel and nonobvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to a nucleic acid encoding an oscillogenin according to the present invention.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl, 0.0015 M sodium titrate, 0.1% SDS at 50°C.; or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42°C. in 0.2X SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal or use materials and methods as described in MANIATIS ET AL., MOLECULAR CLONING: A LABORATORY MANUAL (1989).

As used herein, a nucleic acid molecule is said to be "isolated" or "purified" when the nucleic acid molecule is substantially separated from contaminant nucleic acids encoding other polypeptides.

The present invention further provides fragments of the encoding nucleic acid molecule. As used herein, a "fragment of an encoding nucleic acid molecule" refers to a small portion of the entire protein encoding nucleic acid sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen to encode an active portion of the protein, the fragment will need to be large enough to encode one or more biologically active region(s) of the protein. If the fragment is to be

used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of the encoding nucleic acid molecules of the present invention (*i.e.*, synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding proteins of the invention can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci *et al.*, (*J. Am. Chem. Soc.* 103: 3185-91 (1981)) or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the gene, followed by ligation of oligonucleotides to build the complete modified gene.

The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides and the like. A skilled artisan can employ any of the art known labels to obtain a labeled nucleic acid molecule.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence encoded by a nucleic acid falling within the contemplated scope of the present invention.

Essentially, a skilled artisan can readily use the amino acid sequence of oscillogenin to generate antibody probes to screen expression libraries prepared from appropriate cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described below) or monoclonal antibodies can be used to probe a mammalian cDNA or genomic expression library, such as *λgtll*, to obtain the appropriate coding sequence for other members of the protein family. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme.

Alternatively, a portion of the coding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the oscillogenin family of proteins from any organism. Oligomers containing approximately about 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives. Oligomers can also be prepared which encode about 8, 9, 10, 15 or more consecutive amino acids of oscillogenin.

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively clone an encoding nucleic acid molecule. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other oscillogenin encoding nucleic acid molecules, or as described in NEWTON ET AL., PCR (1997).

***Recombinant Oscillogenin.*** The present invention further provides recombinant DNA molecules (rDNAs) that contain a coding sequence. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in situ*. Methods for generating rDNA molecules are well known in the art, for example, see SAMBROOK *ET AL.*, CLONING: A LABORATORY MANUAL (1989). In the preferred rDNA molecules, a coding DNA sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which an oscillogenin encoding sequence is operably linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the structural gene included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a coding nucleic acid molecule will include a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene, whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or bacteriophage promoter capable of directing the expression (transcription and translation) of the coding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pPL and pKK223 (Pharmacia; Piscataway, N.J.).

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to form a rDNA molecules that contains a coding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), and pTDT1 (ATCC, #31255), and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene (Southern *et al.*, *J. Mol. Anal. Genet.* 1: 327-41 (1982)). Alternatively, the selectable marker can be present on a separate plasmid, and the two

vectors are introduced by co-transfection of the host cell, and selected by culturing cells with the appropriate drug for the selectable marker.

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a protein of the invention are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human cell line. Preferred eukaryotic host cells include Chinese hamster ovary (CHO) cells (ATCC No. CCL61), NIH Swiss mouse embryo cells NIH/3T3 (ATCC No. CRL 1658), baby hamster kidney cells (BHK), fibroblasts and similar eukaryotic tissue culture cell lines.

Any prokaryotic host can be used to express a rDNA molecule encoding a protein of the invention. A preferred prokaryotic host is *E. coli*.

Transformation or transfection of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically used, see, for example, Cohen *et al.*, *Proc. Natl. Acad. Sci. USA* 69: 2110, (1972); and MANIATIS *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982) and SAMBROOK *ET AL.*, (1989). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically utilized, see, for example, Graham *et al.*, *Virol.* 52: 456-67 (1973); Wigler *et al.*, *Proc. Natl. Acad. Sci. USA* 76: 1373-76 (1979).

Successfully transformed cells, *i.e.*, cells that contain a new nucleic acid molecule (*e.g.*, rDNA) of the present invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA or RNA

content examined for the presence of an oscillogenin nucleic acid using a method such as that described by Southern, *J. Mol. Biol.* 98: 503-17 (1975) or Berent *et al.*, *Biotech.* 3: 208 (1985) or the proteins produced from the cell assayed via a suitable immunological detection method.

***Recombinant Oscillogenin Protein.*** The present invention further provides methods for producing a protein of the invention using recombinant nucleic acid molecules herein described. In general terms, the production of a recombinant form of a protein typically involves the following steps:

First, a nucleic acid molecule is obtained that encodes oscillogenin protein of the invention. The coding sequence, preferably lacking introns, is directly suitable for expression in any host. The sequence can be transfected into host cells, such as eukaryotic cells or prokaryotic cells. Eukaryotic hosts include mammalian cells, as well as insect cells (*e.g*, Sf9 cells) using recombinant baculovirus. Alternatively, fragments encoding only portion of oscillogenin can be expressed alone or in the form of a fusion protein. The fusion proteins can be purified and used to generate polyclonal antibodies.

The nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing open reading frame (ORF) of oscillogenin. The expression unit is used to transform a suitable host, and the transformed host is cultured under conditions that allow the production of the recombinant protein. Optionally, the recombinant protein is isolated from the medium or from the cells. Recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any

host/expression system known in the art for use with the nucleic acid molecules of the invention to produce the desired recombinant protein or polypeptide.

## IV. Method of Parthenogenetically Activating Oocytes Using Oscillogenin for Nuclear Transfer

An important embodiment of the invention is directed to use of oscillogenin for parthenogenetic activation of oocytes. Such activation can occur by (1) administering oscillogenin alone or in combination with other $Ca^{2+}$ oscillating agents, and (2) administering oscillogenin in combination with a sperm or a somatic cell. Oscillogenin can be used in combination with sperm for intracytoplasmic sperm injection (ICSI), sperm fertility testing (*e.g.*, efficacy of oscillogenin augmentation), and for *in vitro* fertilization (IVF).

***In vitro fertilization procedures.*** Fertilization procedures can be used as described as in Long *et al.*, *Mol. Reprod. Dev.* 36: 23-32 (1993); Alan O. Trounson *et al.*, HANDBOOK OF IN VITRO FERTILIZATION (1999); and Brigid Hogan *et al.*, MANIPULATING THE MOUSE EMBRYO: A LABORATORY MANUAL (Cold Spring Harbor Laboratory, 1994). Typically, for example, pooled semen, which even can be cryopreserved, is processed using the Percoll method (Hossain *et al., Arch. Androl.* 37: 189-95 (1996)). The separated motile sperm are added at a final concentration of 500,000 sperm/ml. Heparin (10 μg/ml; Sigma) is added to the fertilization medium to induce sperm capacitation (Parrish *et al.*, *Biol. Reprod.* 38: 1171-80 (1988)). Eggs are incubated with sperm for at least 4 hours before monitoring. Eggs that subsequently exhibit $[Ca^{2+}]_i$ oscillations are fixed and stained to confirm fertilization. The fixation and staining procedures and the criteria used to classify the fertilization stages of a zygote are as described by Fissore *et al.*, *Biol. Reprod.* 47: 960-9 (1992) and Long *et al.*, (1993).

***Medium, Calcium Ionophores, Phosphatases and Protein Kinase Inhibitors.*** In addition to injecting oscillogenin into an enucleated oocyte or a nucleated oocyte, microinjected oocytes, nuclei from another cell, the cells also can be incubated in a medium enriched with calcium ions ($Ca^{2+}$). Alternatively, or in addition to culturing in $Ca^{2+}$ enriched medium, the oocyte can be coinjected with or exposed to calcium ionophores (*e.g.*, ionomycin and A23187), protein kinase inhibitors (*e.g.*,

6-dimethylaminopurine (DMAP), butyrolactone, roscovitine, p34(cdc2) inhibitors, staurosporine, 2-aminopurine and sphingosine or other serine-threonine kinase inhibitors) or phosphatases (*e.g.*, phosphatase 2A or phosphatase 2B) to enhance the calcium oscillations in the cell (*e.g.*, oocyte). Incubation in calcium ion enriched mediums can be carried out as described by Wang *et al., Mol. Reprod. Dev.* 53: 99-107 (1999). Also, other divalent cations can be utilized to activate at least rodent oocytes, such as magnesium, strontium, and barium. Divalent cation levels can also be increased using electric shock, oocyte treatment with ethanol and treatment of oocytes with caged chelators.

Calcium ionophores are typically used in combination with protein kinase inhibitors. Embodiments of this invention contemplate use of either an ionophore and oscillogenin or a protein kinase inhibitor and oscillogenin, or all three. Protein kinase inhibitors can be utilized as described in U.S. Patent No. 5,945,577. Calcium ionophores, in combination with protein kinase inhibitors, can be used as described in Susko-Parrish *et al., Dev. Biol.* 166: 729-39 (1994); Mitalipov *et al.* , *Biol. Reprod.* 60: 821-7 (1999); Liu *et al., Biol Reprod.* 61: 1-7 (1999); Mayes *et al., Biol. Reprod.* 53: 270-5 (1995); and U.S. Patent No. 5,496,720. Typically, oocytes are briefly (*e.g.*, approximately 5 minutes) exposed to the ionophore. Phosphatases also can be used to increase calcium levels as described in U.S. Patent No. 5,945,577.

***Parthenogenetic Activation of Oocytes.*** Parthenogenetic activation of oocytes can be induced several ways including: (1) basic treatment with a Ca-ionophore and cytochalasin D combined with cycloheximide; (2) electric impulse; (3) cycloheximide and electric pulse treatments (see Bodo *et al., Acta Vet. Hung* 46: 493-500 (1998)); (4) combined use of calcium ionophores (*e.g.*, A23187) and protein kinase C stimulators (*e.g.*, phorbol esters) (Uranga *et al., Int'l. J. Dev. Biol* . 40: 515-9 (1996)); (5) oocyte exposure to 7% (v/v) ethanol solution (Lai *et al., Reprod. Fertil. Dev.* 6: 771-5 (1994)); (6) induction using puromycin (De Sutter *et al., J. Assist. Reprod. Genet.* 9: 328-37 (1992)); (7) incubation of oocytes in strontium ion enriched medium (O'Neill *et al., Mol. Reprod. Dev.* 30: 214-9 (1991)); and (8) 200 μm thimerosol, which has been observed to induce $Ca^{+2}$ oscillation in pig oocytes (Machaty *et al., Biol. Reprod.* 57: 1123-7 (1997)). In addition to methods of inducing parthenogenetic activation, the efficacy of

activation can be affected by cryopreservation of the oocytes (see, *e.g.*, Lai *et al.*, *Reprod. Fert. Dev.* 6: 771-5 (1994)). Consequently, another embodiment of this invention is to compensate for lower parthenogenetic efficiencies induced by cryopreservation, as well as to provide new materials of improving overall efficacy of parthenogenetic activation of oocytes using freshly harvested cells.

## V. Method of Enhancing ICSI

Another embodiment of the application is to use oscillogenin to enhance the ICSI efficacy in both animal husbandry and *in vitro* fertilization (IVF). Oscillogenin can be used alone with the ICSI technique, or in combination with one or more calcium ionophores, protein kinase inhibitors, phosphatases or calcium enriched mediums, as discussed above. To further enhance sperm-oocyte fusion, electrical stimulation can be utilized as described by Yanagida *et al.*, *Hum. Reprod.* 14: 1307-11 (1999)).

Another method which can be used in combination with those listed above is the vigorous aspiration of oocyte cytoplasm to improve ICSI outcomes as described by Tesarik *et al.*, *Fertil. Steril.* 64: 770-6 (1995).

***Kinase Assays.*** Kinase assays can be used to determine if oscillogenin-induced $[Ca^{2+}]_i$ oscillations are capable of evoking oocyte activation, and thus determine the efficiency of each of the above combinations of techniques and compositions. Suitable kinase assays include histone H1 and mitogen-activated protein (MAP) kinase assays, which can be performed as described by Fissore *et al.*, *Biol. Reprod.* 55: 1261-70 (1996). Myelin basic protein (MBP) is assumed to measure mostly MAP kinase activity, as shown previously (*Id.*). Groups of five eggs are transferred into 5 µl of an H1 kinase buffer solution containing 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 500 nM protein kinase A inhibitor, 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl, and 1 mM dithiothreitol (DTT) (as described by Collas *et al.*, *Mol. Reprod. Dev.* 34: 224-231 (1993)). Eggs are lysed with repeated cycles of freezing and thawing and stored at -80°C until the kinase assay is performed.

Kinase reactions are started by adding 5 µl of a solution containing 2 mg/ml histone H1 (type III-S, Sigma), 1 mg/ml MBP (Sigma), 0.7 mM ATP, and 50 µCi of [$\gamma$-$^{32}$P] (Amersham, Arlington Heights, IL) to 5 µl of the crude egg lysates. The reaction

is carried out for 30 min. at 30° C. and terminated by the addition of 5 µl of SDS sample buffer (Laemmli, *Nature* 227: 680-685 (1970)). Samples are boiled for 3 min. and loaded onto about a 12 or 15% SDS-polyacrylamide gel. Control samples typically contain all the components for the reaction except oocytes. Phosphorylation of histone H1 and MBP is visualized by autoradiography using DuPont's Cronex intensifying screens at -70°C. or other similar system. Such kinase assays can be used to assess sperm specimen fertility, as well as assess the efficacy of a specific combination of techniques and/or compositions. Other conditions for performing the kinase assay would be known to the skilled artisan.

Additional methods to determine whether the oocyte has been induced into a pathway of fertilization, at least in rodents, can be determined by whether the second polar body is extruded. Extrusion of the second polar body can be visualized via microscopy. Also, down-regulation of the inositol triphosphate receptor ($IP_3R$) only appears to occur following fertilization, SF injection and inositol triphosphate ($IP_3$) injection, but not when oocytes are exposed to ethanol, calcium ionophores or strontium chloride. Down-regulation of $IP_3R$ can be assessed both at the level of RNA transcription or at protein synthesis. Such methods are commonly known in the art, for example see ED HARLOW *ET AL.*, ANTIBODIES: A LABORATORY MANUAL (1988); and SAMBROOK *ET AL.*, CLONING: A LABORATORY MANUAL (1989).

## VI. Method and Kit for Assessing Sperm Fertility

Another embodiment of the invention is to measure the oscillogenin content of sperm as a means of measuring sperm fertility. The content can be measured by detecting the concentration and/or localization of oscillogenin in sperm. Oscillogenin can be assessed using antibodies or immunogenic fragments thereof that recognize and bind to oscillogenin. Alternatively, oscillogenin can also be assessed using nucleic acid probes that detect mRNAs. These procedures can be performed using any of the following techniques or as described in the examples.

In one embodiment, the present invention provides a method for determining fertility, by measuring the presence and concentration of oscillogenin in the sperm of the animal being tested. In this method the presence or absence of oscillogenin in a sperm

sample is assayed by measuring the amount, if any, of oscillogenin in the sperm from the sample which binds to an anti-oscillogenin antibody. The anti-oscillogenin antibody can be polyclonal, but is preferably monoclonal. Oscillogenin can also be identified the monoclonal antibody of the present invention.

Typically in domesticated animals, there are about $1x10^9$ sperm/ml of ejaculate. Fertility of an animal can then be determined by screening the collected sample for the presence and amount of oscillogenin. Testing using antibodies can be performed using Western blot assays, ELISAs and other immune assays as would be known to the skilled artisan.

***Enzyme Linked Immunosorbent Assay (ELISA).*** A preferred immunologic means of detecting oscillogenin is the ELISA method. A protein sample is then contacted with these plates. The samples are preferably prepared by diluting oscillogenin removed from a known number of sperm in an incubation-suitable buffer. The samples are placed in the well, incubated at a temperature ranging from about 25°C. to about 37°C., and preferably at about 37°C. for a time period of from about 1 hour to about 4 hours, and preferably about one hour. The wells containing the sample are washed thoroughly before introducing a detection antibody (*e.g.*, anti-oscillogenin antibodies) into the well.

An antibody can be directly labeled or detected using a second antibody. The label may suitably be any which is conventionally attached to monoclonal antibodies or antibody fragments for use in an immunoassay, such as an enzyme (*e.g.*, horseradish peroxidase), a chromophore, a fluorophore (*e.g.*, green fluorescent protein, blue fluorescent protein, or luciferase), or a radiolabel (*e.g.*, $^{125}I$). The label may be bonded to the monoclonal antibody by any conventional method including via conventional cross-linking agents. See ED HARLOW *ET AL.*, ANTIBODIES: A LABORATORY MANUAL (1988).

***Western Blot and Immunoprecipitation.*** For assessing steady-state protein concentrations, Western blots can be used. For Western blots, a typical procedure can be performed as follows. Equal number of spermatozoa contained in, for example, 200 µl of semen is added to a 1.5 ml microcentrifuge tube with 1 ml phosphate buffered saline (PBS) containing TWEEN and 1% bovine serum albumin (BSA) and protease inhibitors, and centrifuged at 4000 rpm to remove seminal fluid. The sperm can be washed 2-3

times before adding sample buffer (Laemmli, 1970) and boiling for 5 min prior to being applied to either 10-15%, preferably 12%, polyacrylamide gels, transferred and Western blotted.

For immunoprecipitation, a typical procedure can be performed by conjugating a monoclonal antibody to HZ Beads (Sigma Chemical Co., St. Louis, Mo.) or similar beads. The membranes of washed sperm are lysed with detergent or with mechanical means and then removed by centrifugation. The beads are added to the supernatant and protein is allowed to bind to the antibody (~10 min). The beads are then washed three times, boiled in sample buffer and the sample buffer is applied to 10-15%, preferably 12%, PAGE. The presence of protein can be determined directly using any suitable protein assaying technique such as Coomassie blue staining of the gels, ELISA or by Western blot. Other methods of immunodetection are as described in HARLOW *ET AL.*, (1988).

***Immunofluorescence.*** Antibodies which recognize and bind to oscillogenin can be used in conjunction with secondary antibodies with fluorescent tags. The fluorescent tags can be fluorescein, rhodamine, rhodamine GREENÔ and other like fluorescent labels.

***Electron microscopic analysis.*** In another embodiment of the invention, electron microscopy can be used to assess the concentration and location of oscillogenin in spermatozoa. Spermatozoa can be fixed for electron microscopy by the procedure described by R. C. Jones, *J. Reprod. Fertil.* 193: 145-149 (1973).

## VII. Method and Compositions for Modulating Oscillogenin Activity

An embodiment of the invention involves compositions and methods of modulating oscillogenin activity and thereby sperm fertility and/or oocyte activation.

For example, oscillogenin can be administered into a targeted oocyte either alone or in combination with (1) a sperm or its genetic material or (2) a somatic cell or its genetic material. When oscillogenin is administered in combination with for example, a sperm, oscillogenin can be administered prior to, simultaneously with, or immediately after injection of, for example, a sperm. Oscillogenin can further be administered with any of the agents which regulate calcium ion oscillations.

## VIII. Antibodies

Another embodiment of the invention is antibodies or immunogenic fragments which recognize and bind to oscillogenin. Anti-oscillogenin antibodies are prepared by immunizing suitable mammalian hosts using appropriate immunization protocols and oscillogenin or immunogenic peptides thereof. These peptides can be at least 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40 or 50 consecutive amino acids in length, or the entire oscillogenin protein. Oscillogenin or an immunogenic fragment thereof, may be conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be desirable to provide accessibility to the hapten. The hapten peptides (such as the lengths described above) can be extended at either the amino or carboxy terminus with a Cys residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier.

Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

Anti-peptide antibodies can be generated using synthetic peptides corresponding to, for example, the amino or carboxy terminal 15-20 amino acids of oscillogenin. Synthetic peptides can be as small as 2-3 amino acids in length, but are preferably at least about 4 to about 20 or more amino acid residues long. The peptides are coupled to KLH using standard methods and can be immunized into animals such as rabbits. Other animals such as rodents (*e.g.*, mice), sheep, goats, horses and other ungulates may also be used. Polyclonal anti-oscillogenin antibodies or peptide antibodies can then be purified, for example using Actigel beads containing the covalently bound peptide or protein.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal (mAb) preparations is preferred. Immortalized cell lines, which secrete the desired monoclonal antibodies, may be prepared using the standard method of Kohler and Milstein (*Nature* 256: 495-7 (1975)) or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known (*see, e.g.*, Harlow *et al.* 1988). The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten, polypeptide or protein. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonal or the polyclonal antisera, which contain the segment which recognizes and binds to oscillogenin, can be used to label oscillogenin as well as potential regulators of oscillogenin. Use of immunologically reactive fragments, such as the Fab, SCFV, Fab', of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

## EXAMPLES

### Example 1

### Method of Enriching Oscillogenin

The following procedure can be used to enrich the sperm factor (SF), a $Ca^{2+}$-release activating protein, by sequential chromatography and to identify the effector polypeptide by comparative SDS-polyacrylamide gel electrophoresis (PAGE).

To fractionate boar sperm factor (SF) by sequential chromatography and to identify the candidate polypeptide the procedures are:

a) Precipitation with ammonium sulfate. The active material precipitates at 50% saturated solution and provides a 2-fold enrichment of the $[Ca^{2+}]_i$-releasing activity.

b) Affinity chromatography on HiTrap blue dye. The active polypetide elutes as peak no. 3 with 1 M KCl. This gives an enrichment of 4-fold.

c) Hydroxyapatite chromatography. Elution from this column is accomplished with increasing concentration of phosphate buffer. The active protein elutes with peak no. 3 (185 mM potassium phosphate) and provides a 4-fold enrichment of activity.

d) Size exclusion chromatography on Superose 12. The active protein elutes with peak 4 and has an MW of 43 kDa. This gives an enrichment factor of 5-fold.

If each of these procedures works independently, the predicted enrichment when used in combination is approximately 128-fold. As the four procedures were based on distinct biochemical properties, it is highly likely that each depletes a distinct set of boar SF proteins. The 50% ammonium sulfate pellets were accumulated from 12 separate boar ejaculates. The equivalent of one boar ejaculate was solubilized and processed through the blue affinity column. The peak no. 3 proteins were collected and stored at -80°C, which fully preserves activity. This process was repeated 12 times. The peak no. 3 fractions from the 12 runs were then combined and loaded onto the hydroxyapatite column. Then peak no. 3 from the hydroxyapatite column was collected, concentrated and immediately poured onto Superose 12. Each individual fraction (250 µl) in the active peak (peak no. 4) of this column were separately concentrated and tested for $[Ca^{2+}]_i$ releasing activity.

Our results show that several proteins within fraction 4-2, and which exhibit a MW in between 35-80 kDa, could be involved in the ability of SF to trigger $Ca^{2+}$ release.

***Ammonium sulfate precipitation.*** Crude sperm extracts were mixed with saturated ammonium sulfate to 50% saturation. The precipitates were collected by centrifugation (10,000xg, 15 min., at 4°C.) and the pellets were stored at -20°C. until used. Pellets were resuspended in injection buffer (75 mM KCl and 20 mM HEPES, pH=7.0), washed in the same buffer, and concentrated using Centricon-30 ultrafiltration membranes before assaying for $Ca^{2+}$ releasing activity.

***Chromatography.*** Columns (all from Phamacia; Pitscataway, NJ) used in the isolation procedures with fast protein liquid chromatography (FPLC) were utilized according to Reduth *et al.*, *J. Eukaryot. Microbiol.* 41: 95-103 (1994); Morgan *et al.*, *Molec. Biochem. Parasitol* 57: 241-52 (1993); Muranjan *et al.*, *Infect. Immun.* 65: 3806-14 (1997); Wu *et al.*, *Dev Biol.* 203: 369-81 (1998)). The pumps and tubing that serve the chromatographic system were flushed with absolute ethanol and subsequently with sterile PBS prior to all fractionation. Columns were sterile and are stored with azide to prevent contamination. All buffers were autoclaved and filtered before use. Collection tubes were sterile and coated with silicon to reduce non-specific loss of protein. The FPLC, fraction collector and all buffers were housed in a 4°C. room to further reduce bacterial growth and enzyme activity.

***HiTrap blue affinity FPLC chromatography.*** Ammonium sulfate pellets were diluted into buffer A (20 mM HEPES, 1 mM EDTA, pH=7.0), and loaded onto a 5 ml HiTrap Blue affinity column (Pharmacia) by using the FPLC system at 4°C. After a 15 ml wash with buffer A, proteins were eluted with a 20-ml linear gradient from 0 to 500 mM KCl, and finally with a 20-ml 1 M KCl. The activity was observed in peak no. 3 (see Fig. 1A). This fraction was concentrated (12 peaks will be accumulated), washed, and poured onto the hydroxyapatite column.

***Hydroxyapatite FPLC chromatography.*** Proteins from peak no. 3 obtained using the HiTrap blue affinity FPLC chromatographic column were diluted into 10 mM potassium phosphate buffer (pH=6.8) with 200 μM phenylmethanesulphonyl fluoride (PMSF) and loaded at 0.4 ml/min onto a 5 ml hydroxyapatite column using FPLC system at 4°C. After 10 ml wash with 10 mM phosphate buffer, proteins were eluted at the same flow rate by increasing the molarity of the potassium phosphate buffer (pH=7.2 with 200 μM PMSF) in a step-wise manner. The potassium phosphate concentration in each step was as follows: 88 mM, 127 mM, 185 mM, 244 mM, 302 mM and 400 mM. Fractions in peak no. 3 (Wu *et al.*, *Dev. Biol.* 203: 369-81 (1998)) were collected, washed, concentrated and poured onto a Superose 12 column.

***Superose 12 FPLC chromatography.*** The active fractions from hydroxyapatite column (peak 3 concentrated to a total volume less than 250 μl) are loaded at 4°C. onto a Superose 12 HR 10/30 column connected to a FPLC system. Proteins were eluted with

buffer (75 mM KCl and 20 mM HEPES, pH=7.0) containing 200 μM PMSF at a flow rate of 0.1 ml/min and detected at $OD_{280}$ by an UV-M monitor. Each individual fraction (0.25 ml) was collected, and concentrated before testing for $Ca^{2+}$ releasing activity. The Superose 12 HR 10/30 column was calibrated using ß-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa) and carbonic anhydrase (29 kDa) (Sigma).

Example 2

Characterization of the Oscillogenin Protein

This example characterizes the activity of an SF extract

***Egg recovery and culture***

Mouse eggs or recently fertilized zygotes were recovered from the oviducts of 8-20 week old CD-1 female mice as previously described (Wu *et al., Dev. Biol.* 203, 369-81 (1998)). Mice were superstimulated with an injection of 5 I.U. pregnant mare serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, MO; all reagents from Sigma unless specified), and induced to ovulate 40-48 hr later by injection of 5 I.U. human chorionic gonadotropin (hCG; Sigma). To obtain fertilized zygotes, females were placed overnight with males following the injection of hCG. Eggs were collected 14 hr post-hCG (phCG) injection into a HEPES-buffered solution (TL-Hepes) supplemented with 5% heat-treated fetal calf serum (FCS; Gibco, Grand Island, NY). Granulosa cells were removed by a 5-10 min incubation with bovine testis hyaluronidase, and oocytes showing no signs of degeneration and first polar body extrusion were selected for these studies. Eggs were transferred to 50 μl drops of KSOM (Specialty Media, Phillipsburg, NJ), where they were incubated before and after activation for variable periods of time under paraffin oil at 36.5°C in a humidified atmosphere containing 7% $CO_2$ in air.

***Microinjection Techniques***

Microinjection procedures were carried out as previously described (Wu *et al., Dev. Biol.* 203: 369-81 (1998)). Briefly, eggs were placed in a 50 μl microdrop of TL-Hepes supplemented with 2.5% sucrose and 20% FCS under paraffin oil and injected using manipulators (Narishige, Medical Systems Corp., Great Neck, NY) mounted on a Nikon Diaphot microscope (Nikon, Inc., Garden City, NY). Injection pipettes were

loaded by suction from a 2-3 µl drop containing one of the following compounds: 0.5 mM fura-2 dextran (fura-2 D; Molecular Probes, Eugene, OR), 1 mg/ml protein concentration of boar sperm fractions (SF), or 10 µM adenophostin A, a powerful $IP_3R$ agonist (courtesy of Dr. K. Tanzawa, Sankyo CO, Tokyo, Japan). All reagents were diluted in buffer containing 75 mM KCl and 20 mM HEPES, pH 7.0 and were delivered into the ooplasm by pneumatic pressure using a PLI-100 picoinjector (Medical Systems Corp.). Injection volumes were approximately of 5-10 pl, and this resulted in intracellular concentrations of approximately 10 ng/µl for SF (2.5-5 sperm equivalents; Wu *et al.*, 1998), and 100 nM for adenophostin A.

***SF Preparation***

Cytosolic SF extracts were prepared from boar semen as described by Swann, *Development* 110: 1295-1302 (1990); and Wu *et al., Dev. Biol.* 203: 369-81 (1998). In brief, semen samples were first washed twice with TL-Hepes, and the pellet resuspended in a solution containing 75 mM KCL, 20 mM HEPES, 1 mM EGTA, 10 mM glycerophosphate, 1 mM DTT, 200 µM PMSF, 10 µg/ml pepstatin, and 10 µg/ml leupeptin, pH 7.0. The sperm suspension was lysed by sonication (XL2020, Heat Systems Inc., Farmingdale, NY) using a small probe at a setting of 3 for 15-25 min at 4°C. The sonicated suspension was spun twice at 10,000 xg, the supernatant collected both times, and then centrifuged at 100,000 xg for 45 min at 4°C. The resulting clear supernatant was collected as the cytosolic fraction. Ultrafiltration membranes (Centricon-50, Amicon, Beverly, MA) were used to wash the supernatants (75 mM KCl and 20 mM HEPES, pH 7.0). The extracts were then precipitated by exposure to a saturated solution of ammonium sulfate (50% final concentration), followed by centrifugation at 10,000 g for 15 min at 4°C. The precipitates were collected and stored at -80°C until the time of use.

***Parthenogenetic Activation***

Several commonly used parthenogenetic agents were used during the course of these studies, including ethanol and ionomycin, which induce a single $[Ca^{2+}]_i$ rise (Cuthbertson, *J. Exp. Zool.* 226: 311-14 (1983); and Shiina *et al., J. Reprod. Fert* 97: 143-50 (1993)), and others such as adenophostin A, SF, thimerosal, and $SrCl_2$ that evoke $[Ca^{2+}]_i$ oscillations (Kline *et al., Dev. Biol.* 149: 80-9 (1992); Swann, *Biochem J.* 287:

79-84 (1992); and Sato *et al., Biol. Reprod.* 58: 867-73 (1998)). The activating compounds were either injected into eggs (*e.g.*, adenophostin A and SF, see microinjection procedures for details) or added to the eggs' culture media (*e.g.*, thimerosal, $SrCl_2$). Activation was started in all cases at 16 hr post hCG administration (phCG) and eggs evaluated visually 2 hr later by observing second polar body extrusion (18 hr phCG) and 5 hr post-treatment by evaluation of pronuclear formation. After the activation procedure and the assigned incubation period (1, 2, 4 or 8 hr), eggs were collected in 5 µl Dulbecco's phosphate buffered solution (DPBS)/polyvinylpyrrolidone (3 mg/ml, PVP) and stored at -80°C. The great majority of eggs collected 8 hr post-treatment, except those treated with thimerosal, exhibited pronuclear formation.

Ethanol activation was carried out by exposing eggs to a 7% ethanol solution in TL-Hepes plus 3 mg/ml bovine serum albumin (BSA) for 5 min at 37°C. After the treatment, eggs were washed several times in TL-Hepes, and cultured for 8 hr (24 hr-phCG). Activation with ionomycin and 6-dimethylaminopurine (DMAP), a kinase inhibitor (Susko-Parrish *et al., Dev. Biol.* 166: 729-39 (1994)) was accomplished by incubating eggs with 5 µM ionomycin for 5 min in $Ca^{2+}$ free-DPBS plus 3 mg/ml BSA. Eggs were then washed in TL-Hepes/1mg/ml BSA, placed in DMAP/KSOM (2 mM) for 4 hr and cultured for 4 hr after carefully washing them free of DMAP. Eggs activated with $SrCl_2$ were incubated for 2 or 4 hr in a $Ca^{2+}$-free M-16-like medium supplemented with 10 mM $SrCl_2$. The treated eggs were then washed in TL-Hepes supplemented with 5% FCS and cultured for 2, 4 or 6 hr, depending on the experiment. For thimerosal activation, eggs were exposed to freshly made solutions of 200 µM thimerosal in KSOM for 30 min. Thimerosal-treated eggs were washed free of the reagent using TL-Hepes supplemented with 5% FCS and incubated for 30 min, 1.5, 3.5 or 7.5 hr.

***Fluorescence Recordings and $[Ca^{2+}]_i$ Determinations***

Monitoring of $[Ca^{2+}]_i$ levels using fura-2 D loaded eggs was carried out as previously described (Wu *et al.*, 1998). UV illumination was provided by a 75 watt xenon arc lamp, and 340 and 380 nm excitation wavelengths were utilized. The intensity of the UV light was attenuated 32-fold with neutral density filters, and a photomultiplier tube quantified the emitted light after passing through a 500 nm barrier filter. The fluorescence signal was averaged for the whole egg. A modified Phoscan 3.0 software program run on a 486 IBM-compatible system controlled the rotation of the filter wheel and shutter apparatus to alternate wavelengths. Free $[Ca^{2+}]_i$ was determined from the 340 nm/380 nm ratio of fluorescence. $R_{min}$ and $R_{max}$ were calculated using 10 μM fura-2 D in $Ca^{2+}$-free DPBS supplemented with 2 mM EDTA ($R_{min}$) or 2 mM $CaCl_2$ ($R_{max}$) and with 60% sucrose to correct for intracellular viscosity (Grynkiewicz *et al., J. Biol. Chem.* 260: 3440-50 (1985); and Poenie, *Cell Calcium* 11: 85-91 (1990)). The same solutions were also used alone for background subtractions. $Ca^{2+}$ measurements carried out in the presence of extracellular $SrCl_2$ are presented as fluorescence ratios of the 340 nm/380 nm excitation wavelengths. Calculations of the $[Ca^{2+}]_i$ concentration was in this case not performed, since fura-2 has also some affinity for $Sr^{2+}$. The presence of intracellular $Sr^{2+}$ therefore interferes and prevents obtaining accurate $Ca^{2+}$ and/or $Sr^{2+}$ intracellular concentrations (Hajnoczky *et al., EMBO J.*, 16: 3533-43 (1997)).

Eggs were measured individually in 35 μl drops of TL-Hepes medium placed on a glass coverslip on the bottom of a plastic culture dish under paraffin oil. Fluorescence ratios were measured every 6 sec, and readings were taken for 1 sec at each wavelength. Oocytes were first monitored for 10-120 sec to establish baseline $[Ca^{2+}]_i$ values, after which, recordings were stopped for 2-6 min to allow for microinjection or addition of reagents. Recordings were then restarted and continued for 10-30 min.

***Inhibitor Preparation***

Lactacystin (100 μM; Calbiochem, La Jolla, CA), a proteasome inhibitor (Mellgren, *J. Biol. Chem.* 272: 29899-903 (1997); and Fenteany *et al., J. Biol. Chem.* 273: 8545-48 (1998)), was used to determine if the proteasome was involved in down-regulation of the $IP_3R$-1 by SF. Eggs were incubated in the inhibitor for 30 min. prior to injections, and injections of SF were carried out in the presence of the inhibitor. Eggs

were then cultured in a new drop of KSOM freshly supplemented with lactacystin for 2 hr.

***Western Blot Technique***

Equal volumes of crude lysates from 15 or 20 mouse eggs and double strength sample buffer (Laemmli, *Nature* 227: 680-5 (1970)) were combined as previously described (He *et al., Biol. Reprod.* 57: 1245-55 (1997)). Samples were boiled for 3 min and loaded into a 4% SDS-polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membranes (Micron Separation; Westboro, MA) using a Mini Trans Blot Cell (Bio-Rad; Hercules, CA) for 2 hr at 4°C. The membranes were first washed in PBS and 0.05% Tween (PBS-T) and then blocked in 6% nonfat dry milk in PBS-T for 1 hr. After several washes in PBS-T, the membranes were incubated overnight with a rabbit polyclonal antibody raised against a 15 peptide sequence of the C-terminal end of the $IP_3R$-1 subtype (Rbt04) diluted to 1:3,000 in PBS-T (Parys *et al. Cell Calcium,* 17: 239-49 (1995)). Following several washes, the membranes were incubated for 1 hr with a secondary antibody coupled to horseradish peroxidase and diluted 1:3,000 in PBS-T. The membranes were developed using western blot chemiluminescence reagents (NEN Life Science Products; Boston, MA) and exposed for 1-3 min. to maximum sensitivity film (Kodak, Fisher Scientific; Springfield, NJ). Broad range, pre-stained SDS-PAGE molecular weight markers (Bio-Rad) were run in parallel to estimate the molecular weight of the immunoreactive bands. The intensity of the $IP_3R$-1 bands was quantified using Adobe Photoshop (Mountain View, CA) essentially as described by Cameron *et al., Cell* 83: 463-72 (1995) and plotted using Sigma plot software (Jandel Scientific Software; San Rafael, CA). The mean pixel intensity within a selected set area containing each $IP_3R$-1 band was obtained, and the same set area was applied to all lanes for that particular film. The same set area was also placed in an area of the film where there were no bands, and a background number was taken. This background number was then subtracted from all $IP_3R$-1 densities for the film under consideration. The band from metaphase II (MII) eggs was used as a reference and assigned the value of 1. The intensity of the $IP_3R$-1 band from eggs after treatment with several different parthenogenetic agents was calculated relative to 1 and statistically compared. To avoid possible saturation of the quantification system and to be sure that quantification was

performed in the linear range, 4 or 5 exposures of each film were obtained and quantified. Underexposed and overexposed exposures were discarded. Western blotting procedures were repeated at least three times, and eggs were collected over several different dates.

*Statistical Analysis*

Statistical comparisons of the intensity of $IP_3R$-1 bands and of the $Ca^{2+}$ parameters were performed using one-way ANOVA. If differences were observed between groups, comparisons between treatments were achieved by applying the Tukey-Kramer method using JMP IN software (SAS Institute; Cary, NC). Significance was at $p < 0.05$.

***RESULTS***

***Egg aging and fertilization have dissimilar effects on $IP_3R$-1 down-regulation***

Aging of eggs or their fertilization has been shown to induce a marked decrease in the eggs' $Ca^{2+}$ responses to $IP_3$ injection (Jones *et al., Development* 121: 3259-66 (1995); and Jones *et al., Dev. Biol.* 178: 229-37 (1996)). To demonstrate if the reduced responsiveness of the $IP_3R$-system was due to a decrease in the number of $IP_3R$-1, mouse eggs were either collected after ovulation and aged *in vitro*, or collected soon after fertilization and cultured *in vitro* for a variable period of time. Unfertilized eggs were cultured for 10 hr (24 hr phCG), or 16 hr (30 hr phCG), and zygotes were cultured to the pronuclear stage (24 hr phCG), to immediately before first mitosis (30 hr phCG), or to the 2-cell stage (40 hr phCG). The presence and amounts of $IP_3R$-2 in these eggs was assessed by Western blotting. As shown in Fig. 2A, B, aging of eggs *in vitro* had no significant effect on the amount of $IP_3R$-1. Conversely, fertilization induced marked down-regulation of the $IP_3R$-1 by the time of pronuclear formation (Fig. 2C, D). No significant additional down-regulation of the receptor was observed after this time (Fig. 2C, D).

***Single $[Ca^{2+}]_i$ rises induced by ethanol and ionomycin do not down-regulate $IP_3R$-1***

To understand the mechanism(s) by which $IP_3R$-1 is down-regulated during fertilization, we investigated whether inducing a single $[Ca^{2+}]_i$ rise had an impact on $IP_3R$-1 degradation. It is well established that single $Ca^{2+}$ responses, like those induced by ethanol and ionomycin, trigger high rates of activation and initiation of development

in aged oocytes (Cuthbertson, *J. Exp. Zool.* 226: 311-14 (1983); Shiina *et al., J. Reprod. Fert.* 97: 143-50 (1993); and Susko-Parrish *et al., Dev. Biol.* 166: 729-39 (1994). In addition, DMAP, a kinase inhibitor, was added to test the possibility that in the absence of oscillations, additional kinase activity down-regulation may stimulate $IP_3R$-1 degradation. Thus, we investigated if mouse eggs exposed to 70% ethanol or 5 μM ionomycin+2 mM DMAP and collected at the pronuclear stage (24 hr phCG) exhibited down-regulation of the $IP_3R$-1. Exposure to these agents was unable to signal $IP_3R$-1 degradation (Fig. 3), although they induced high rates of activation. However, fertilization and injection of SF induced down-regulation of the receptor (Fig. 3C, D). These results suggest that $[Ca^{2+}]_i$ oscillations may be required to induce fertilization-like $IP_3R$-1 down-regulation.

***$[Ca^{2+}]_i$ oscillations induced by injection of SF and adenophostin A, but not by exposure to $SrCl_2$, induce $IP_3R$-1 degradation***

To test the notion that multiple $[Ca^{2+}]_i$ rises are required for down-regulation of $IP_3R$-1, oscillations were initiated in mouse eggs by three different compounds that act on different molecular targets of the $Ca^{2+}$ signaling pathway. Injection of SF, which triggers fertilization-like oscillations by presumably stimulating production of $IP_3$ (Jones *et al., FEBS Lett.* 437: 297-300 (1998)), induced marked down-regulation of the $IP_3R$-1 (Fig. 4A, B). The down-regulation of $IP_3R$-1 was persistent, although significant degradation was seen within 1 hr post-injection (Fig. 4A, B). Injection of adenophostin A, a non-hydrolyzable agonist of the $IP_3R$, also induced significant down-regulation of the receptor (Fig 4C, D). Interestingly, this down-regulation was consistently greater than the degradation induced by fertilization ($p < 0.05$) or by injection of SF (Fig. 4C, D). Finally, exposure of eggs to $SrCl_2$ for 2 or 4 hr failed to induce any changes in the amount of $IP_3R$-1 (Fig. 5 A, B). Together, these results suggest that down-regulation of $IP_3R$-1 during mouse egg fertilization is not exclusively due to the presence of multiple $[Ca^{2+}]_i$ rises, but may be associated with $[Ca^{2+}]_i$ oscillations initiated by activation of the phosphoinositide pathway.

***Thimerosal induces $IP_3R$-1 down-regulation***

Thimerosal, a thiol oxidizing agent, has been shown to induce $[Ca^{2+}]_i$ rises without stimulating production of $IP_3$ (Hecker *et al., Biochem. Biophys. Res. Comm.* 159:

961-68 (1989); Bootman *et al., J. Biol. Chem.* 267: 25114-9 (1992); and Missiaen *et al., J. Physiol. London* 455: 623-40 (1992)). Thus, we investigated if oscillations initiated by co-incubation of eggs with this compound induced $IP_3R$-1 degradation. Thimerosal-mediated $Ca^{2+}$ responses induced rapid and sustained down-regulation of the receptor (Fig. 5C, D). These data suggest that $IP_3R$-1 down-regulation in mouse eggs may not be exclusively signaled by activation of the phosphoinositidase pathway.

***Patterns of $[Ca^{2+}]_i$ oscillations are agonist-specific***

Due to the differential effects on $IP_3R$-1 down-regulation by the different agonists tested, we examined the $Ca^{2+}$ responses triggered by each of these agonists. As expected, injection of SF (n=4 eggs) and adenophostin A (n=6 eggs) induced $[Ca^{2+}]_i$ rises similar to those initiated by fertilization, but with higher frequency ($p < 0.05$; Fig. 6A and B, respectively). On the contrary, eggs exposed to $SrCl_2$ (n=5 eggs) exhibited oscillations with lower frequency and these rises were different than those initiated by the other agonists in which the first rise and subsequent rises were very prolonged (Table 1; $p < 0.05$; Fig. 6C). Eggs stimulated with thimerosal (n=9 eggs) showed $Ca^{2+}$ responses with low frequency ($p < 0.05$). However, the amplitude of thimerosal-induced spikes had comparable amplitude to those induced by SF and adenophostin A, although the first rise was of lower amplitude (Table 1; $p < 0.05$). The amplitude of $SrCl_2$-induced rises was not compared to those induced by the other agonists, because fura-2 D, in this study, was calibrated to report intracellular $Ca^{2+}$ levels and it is likely that the observed fluorescence changes represent changes in the concentrations of both cations (Hajnoczky *et al., EMBO J.* 16: 3533-43 (1997)). Despite the different $Ca^{2+}$ profiles, the $Ca^{2+}$ responses induced by all agonists (thimerosal excluded), appeared physiological as more than 90% of the eggs were activated and exhibited pronuclear formation (data not shown).

**TABLE 1**

Characteristics of $[Ca^{2+}]_i$ rises induced by several common agonists in mouse eggs.

| $[Ca^{2+}]_i$ oscillation inducing agonist | # of Eggs | # of rises in 5 min * | Amplitude of 1st rise (nM) | Amplitude of 3rd rise (nM) | Duration of 1st rise (sec) | Duration of 3rd rise (sec) |
|---|---|---|---|---|---|---|
| Adenophostin A | 6 | 3.9±1.5[a]** | 620±30[a,b] | 530±100[a] | 260±65[a] | 70±22[a] |
| SF | 4 | 5.0±1.0[a] | 870±40[a] | 440±35[a] | 330±90[a,b] | 60±18[a] |
| $SrCl_2$ | 5 | 1±0.0[b] | ND*** | ND | 740±290[b] | 360±70[b] |
| Thimerosal | 9 | 1±0.4[b] | 330±30[b] | 640±90[a] | 180±44[a] | 70±16[a] |

* The frequency of oscillations was monitored during a 5 min. period immediately after the return of the first rise to baseline values (Adenophostin A or SF) or after the addition of $SrCl_2$ or thimerosal. All types are means±standard errors of the mean (SEM). The 3rd rise was chosen arbitrarily to represent any subsequent spike in all treatments.

** Values that do not share a common superscript within columns are significantly different ($p < 0.05$).

*** Not determined ("ND"), since $Sr^{2+}$ may bind the fura-2 D and potentially interfere with the correct reporting of intracellular $Ca^{2+}$ values.

***Down-regulation of the $IP_3R$-1 is mediated by the proteasome***

$IP_3R$-1 down-regulation in somatic cells has been shown to be mediated by the proteasome (Bokkala *et al., J. Biol. Chem.*, 272: 12454-61 (1997); and Oberdorf *et al., Biochem J.* 339: 453-61 (1999)). The proteasome is also likely involved in down-regulation of specific proteins that allow fertilized mammalian eggs to exit the MII arrest (Kubiak *et al., EMBO J.* 12: 3773-8 (1993); for review see Whitaker, *Rev. Reproduction* 1: 127-135 (1996)). Thus, we determined if the degradation of $IP_3R$-1 induced by SF injection involved a similar pathway. To accomplish this, eggs were pre-incubated and injected with SF in the presence of lactacystin, a proteasome inhibitor. Activation was allowed to proceed for 2 hr, at which time the injected eggs were removed and prepared

for Western blotting. The 2 hr time point was chosen because, by 1 hr post-injection, significant down-regulation of $IP_3R$-1 was already observed (Fig. 4 A, B). Degradation of the receptor in SF-injected eggs incubated in lactacystin was markedly inhibited (Fig. 7). Furthermore, the effectiveness of the inhibitor on proteasome activity could also be deduced by the finding that cell cycle progression, as assessed by extrusion of the polar body, was clearly delayed in SF-injected eggs pretreated and incubated with the inhibitor (not shown).

***DISCUSSION***

The results of this study in mouse eggs show a) that parthenogenetic activation induced by a single $[Ca^{2+}]_i$ rise initiated by exposure to ethanol or ionomycin/DMAP does not induce down regulation of $IP_3R$-1; b) that initiation of oscillations by injection of SF, adenophostin A, or by exposure to thimerosal, evoked a marked decrease in the levels of $IP_3R$-1 similar to those observed during fertilization; c) that initiation of $[Ca^{2+}]_i$ oscillations by exposure to $SrCl_2$ did not signal $IP_3R$-1 degradation; and d) that down-regulation of $IP_3R$-1 is likely to be mediated by the proteasome, since down-regulation was prevented by lactacystin, a proteasome inhibitor. Together, these data suggest that IPR3-1 down-regulation in mouse eggs after fertilization is associated with activation of the phosphoinositide/$IP_3R$ system and that persistent $IP_3$ production, induced by the sperm during fertilization or by injection of SF in this study, may regulate the degradation of the $IP_3R$-1.

Mammalian oocytes and eggs closely control the number of $IP_3R$-1 before and after fertilization. During oocyte maturation, the increase and redistribution of the $IP_3R$-1 protein is intended to maximize the amount and spatial distribution of $Ca^{2+}$ release following sperm penetration (Fujiwara *et al., Dev. Biol.* 156: 69-79 (1993); Mehlmann *et al., Biol. Reprod.* 51: 1088-98 (1994); and Shiraishi *et al., Dev. Biol.* 170: 594-606 (1995)). However, the role and regulation of the decline of $IP_3R$-1 numbers after fertilization is not fully elucidated, despite the fact that this decline may be specific since it is not observed in unfertilized aged eggs (Parrington *et al., Dev. Biol.* 203: 451-61 (1998) and present data) or in eggs activated by exposure to ethanol or ionomycin. These results indicate that $IP_3R$-1 down-regulation is not an effect of egg activation *per se*, but

may be more closely associated with the number of $[Ca^{2+}]_i$ rises or the mechanism by which the oscillations are initiated, both of which were tested in this study.

$IP_3R$-down-regulation studies in somatic cells have shown that degradation of the receptor requires persistent stimulation of PLC-coupled cell-surface receptors, since activation of these receptors that resulted in brief production of $IP_3$ was unable to induce receptor degradation (Oberdorf *et al., Biochem J.* 339-453-461 (1999)). To produce long-term stimulation in our study, mouse eggs were injected with SF. SF has previously been shown to induce prolonged $[Ca^{2+}]_i$ oscillations that closely mimic those initiated by fertilization (Swann, *Development* 110: 1295-1302 (1990); Wu *et al., Mol. Reprod. & Dev.* 46: 176-89 (1997); and for review see Swann *et al., BioEssays* 19: 79-84 (1997)). These oscillations are mediated by the $IP_3R$ as $Ca^{2+}$ responses were suppressed by injection of the $IP_3R$-1 blocking monoclonal antibody 18A10 (Oda *et al., Dev. Biol.* 209: 172-85 (1999)). In the present study, SF induced a marked and persistent decline of $IP_3R$-1 similar to that observed following fertilization. SF has recently been shown to stimulate production of $IP_3$ in cell-free extracts from sea urchin eggs (Jones *et al., FEBS Letts.* 437: 297-300 (1998)), and thus, it is possible that it may induce $IP_3R$-1 down-regulation by stimulating long-term production of $IP_3$.

Our finding that injections of adenophostin A trigger down-regulation of $IP_3R$-1 supports this hypothesis. Adenophostin A, a product from *Penicillium brevicompactum,* is a full $IP_3R$ agonist that is approximately 100-fold more potent than $IP_3$. Moreover, adenophostin A has greater affinity for the $IP_3R$ and is not degraded by the $IP_3$ metabolizing enzymes (Takahashi *et al., J. Biol. Chem.* 269: 369-72 (1993)). These properties, which may allow this agonist to remain bound to the receptor for longer periods of time, may be responsible for the near total down-regulation of the $IP_3R$-1 observed in adenophostin A-injected eggs. The finding that thimerosal also induces down-regulation of the $IP_3R$-1 suggests that stimulation of the phosphoinositide pathway may not be the only mechanism to signal $IP_3R$-1 degradation in mammalian eggs. Thimerosal, an oxidizing agent that does not trigger $IP_3$ production, has been shown to increase the affinity of $IP_3R$-1 for $IP_3$ (Poitras *et al., J. Biol. Chem.* 268: 24078-82 (1993); Kaplin *et al., J. Biol. Chem.* 269: 28972-8 (1994); and Vanlingen *et al., Cell Calcium* 25: 107-14 (1999)) and it is possible that by this mechanism it may induce

degradation of the $IP_3R$. Alternatively, thimerosal has been demonstrated to oxidize critical cysteine residues in the receptor inducing a change in the conformational state of the $IP_3R$ (Sayers *et al., Biochem. J.* 289: 883-7 (1993)) and, in this manner, it may induce $IP_3R$-1 down-regulation. A conformational change has been shown to occur in the $IP_3R$ following binding of $IP_3$, resulting in the opening of the channel (Mignery *et al., EMBO J.* 9: 3893-9 (1990)). This structural change may also be required for the degradation of the receptor (Zhu *et al., J. Biol. Chem.* 274: 3476-84 (1999)). Therefore, although thimerosal does not stimulate $IP_3$ production, it may signal $IP_3R$-1 degradation by inducing a similar modification of the receptor.

In contrast, $SrCl_2$ failed to induce down-regulation of $IP_3R$-1, despite inducing persistent oscillations. $SrCl_2$ has been suggested to induce $[Ca^{2+}]_i$ oscillations by sensitizing a $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mechanism, although the precise mechanism is not known (Cheek *et al., Development* 119: 179-89 (1993)). The lack of effect of $SrCl_2$-induced responses on $IP_3R$-1 degradation is in marked contrast with the high rates of egg activation induced by this agonist. This demonstrates that $SrCl_2$-induced oscillations are capable of signaling the degradation of specific egg proteins, whose decline is known to be required to exit MII (Whitaker, *Reviews in Reproduction* 1: 127-35 (1996)). This clearly indicates that, in contrast to the situation of other egg proteins, the existence of $[Ca^{2+}]_i$ oscillations and the resulting egg activation are not sufficient to induce down-regulation of $IP_3R$-1.

The conformational change induced by binding of $IP_3$ to its receptor has been suggested to signal $IP_3R$ degradation by enhancing $IP_3R$ ubiquitination and, consequently, signaling degradation by the proteasome (Bokkala *et al., J. Biol. Chem.* 272: 12454-61 (1997); and Oberdorf *et al., Biochem. J.* 339: 453-61 (1999)). In somatic cells, it has been shown that persistent stimulation of the phosphoinosftide pathway results in poly-ubiquitination of the $IP_3R$-1 (Bokkala *et al., J. Biol. Chem.* 272: 12454-61 (1997); and Oberdorf *et al.,* (1999)), and studies using cells expressing mutant $IP_3Rs$-1, which were unable to bind $IP_3$, showed that these receptors were not degraded or ubiquitinated (Zhu *et al., J. Biol. Chem.* 274: 3476-84 (1999)). These studies also demonstrated that ubiquitinated $IP_3Rs$ are degraded by the proteasome as addition of the cysteine protease and proteasome inhibitor, N-acetyl-Leu-Leu-norleucinal, and

lactacystin, a highly specific inhibitor of the proteasome, both blocked the degradation of the receptor (*Wojcikiewicz et al., J. Biol. Chem.* 271: 16652-5 (1996); (Bokkala *et al.*, (1997); and Oberdorf *et al.*, (1999)). Our results in mouse eggs showing that lactacystin blocked down-regulation of $IP_3R$-1 induced by injection of SF is evidence that the proteasome pathway is involved in the decline of $IP_3R$-1 numbers in eggs. Whether $IP_3$ binding to its receptor is the exclusive signal for degradation of the receptor in eggs is not known. $Ca^{2+}$ or protein kinase C (PKC), both of which play a role in activation (Gallicano *et al., BioEssays* 19: 29-36 (1997)), may also participate in signaling $IP_3R$-1 degradation. Our findings that thimerosal, which does not stimulate the phosphoinositide pathway, triggers $IP_3R$ degradation, and that $SrCl_2$, which induces oscillations without affecting receptor degradation, suggest that neither $Ca^{2+}$ nor $Ca^{2+}$-dependent PKC are critical or sufficient for $IP_3R$-1 demise in mouse eggs.

How the decline in $IP_3R$-1 numbers may affect the frequency and duration of $[Ca^{2+}]_i$ oscillations remains to be determined. It is likely, however, that it may be involved in the cessation, or decline in frequency/amplitude, of fertilization/agonist-induced $[Ca^{2+}]_i$ oscillations, which is observed as activated eggs progress to the pronuclear stage (Fissore *et al., Dev. Biol.* 166: 634-42 (1994); Jones *et al., Development* 121: 3259-66 (1995); and Parrington *et al., Dev. Biol.* 203: 451-61 (1998)). It is important to note that concomitant with these changes in $IP_3R$-1, two critical kinase activities also decline in eggs, those of maturation promoting factor and mitogen activated protein kinase (Moos *et al., Biol. Reprod.* 53: 692-9 (1995)). Thus, it will be necessary to determine whether one of these changes is more important than the other in the regulation/cessation of oscillations, or if both contribute equally. The use of eggs/zygotes with different numbers of $IP_3R$-1s but in similar cell-cycle stage/kinase levels, which can now be generated using the different agonists reported in this study, will allow us to discriminate the effect of $IP_3R$ numbers and cell cycle stage on oscillations patterns in mammalian eggs.

In summary, the data presented here show that $IP_3R$-1 down-regulation in mouse eggs is induced by fertilization and by agonists that persistently stimulate the phosphoinositide pathway/$IP_3R$ system. The data also show that the proteasome pathway is likely to mediate the degradation of the $IP_3R$-1.

Example 3

Method of Inducing Parthenogenetic Activation of an Oocyte Using Oscillogenin

Eggs are obtained from the oviducts of a CD-1 female mouse (6-12 weeks old) or other animal, superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma, St. Louis, MO) and is followed by 48 hr later injection of 5 I.U. of human chorionic gonadotropin (hCG; Sigma) to induce ovulation. Eggs are recovered 14 h post-hCG into a Hepes-buffered solution (TL-Hepes; Parrish *et al., Biol. Reprod.* 38: 1171-80 (1988)) supplemented with 10% heat-treated calf serum (CS; Gibco, Grand Island, NY). Cumulus cells are removed with bovine testes hyaluronidase (Sigma).

Microinjection procedures are used as described in Wu *et al., Mol. Reprod. Dev.* 46: 176-89 (1997) and Wu *et al., Mol. Reprod. Dev.* 49: 37-47 (1998). In brief, eggs are microinjected using Narishige manipulators (Medical Systems Corp.; Great Neck, NY) mounted on a Nikon Diphot microscope (Nikon, Inc., Garden City, NY). Glass micropipets are filled by suction of a microdrop containing 0.5 mM fura 2 dextran (fura 2D, dextran 10 kDa, Molecular Probes; Eugene, OR) or sperm extract (1-20 mg/ml protein concentration). Solutions are expelled into the cytoplasm of eggs by pneumatic pressure (PLI-100, picoinjector; Medical Systems Corp., NY). The injection volume is about 5 to about 10 pl and results in final intracellular concentration of the injected compounds of approximately 1% of the concentration in the injection pipette. Injections of sperm factor (SF) results in a $Ca^{2+}$ oscillation and complete activation of oocyte development.

Fura 2D fluorescence is monitored as previously described by Wu *et al.*, (1997 and 1998 above), which monitors $Ca^{2+}$ oscillations. Briefly, excitation wavelengths are at 340 and 380 nm and the emitted light is quantified, after passing through a 500-nm barrier filter by a photomultiplier tube. The intensity of excitation light is attenuated by neutral density filters, and the fluorescent signal is averaged for the whole egg. $[Ca^{2+}]_i$ concentrations ($R_{min}$ and $R_{max}$) are calculated according to Grynkiewickz *et al., J. Biol. Chem.* 260: 3440-50 (1985); Poenie, *Cell Calcium* 11:85-91 (1990); Fissore *et al., Dev. Biol.* 159: 122-30 (1993) and Wu *et al.*, (1997 and 1998). Determining parthenogenetic

activation can be performed by visualization of whether the cell forms a pronucleus or undergoes a first cleavage event. Parthenogenic activation can also be assessed biochemically by assessing whether histone H1 is down-regulated, DNA synthesis is up-regulated, or by other methods which would be known to the skilled artisan.

$[Ca^{2+}]_i$ monitoring for determining parthenogenetic activation of the mouse eggs starts 30-45 min. after injection of fura 2D, which is approximately 15 hr post-hCG administration. Eggs are monitored individually in 50 µl medium placed on a glass coverslip on the bottom of a culture dish covered with paraffin oil. Fluorescence ratios are obtained every 4 sec for 15 to 30 min. Prior to the injection of oscillogenin, fluorescent recordings are taken to establish baseline values. Readings are taken for 1 sec at each wavelength. $[Ca^{2+}]_i$ monitoring is completed before eggs have reached 22 h post-hCG. All sperm extracted fractions are tested at 1 mg/ml protein concentration.

Example 4

An Anti-Oscillogenin Antibody

Antibodies can also be prepared by subcloning the oscillogenin cDNA into a glutathione S-transferase (GST)-gene based expression vector pGEX 3 system. The correct orientation and position of the oscillogenin insert is confirmed by sequencing of nucleotides in the site of transcription initiation. The construct is then transformed into *Escherichia coli* BL21 strain, and GST-oscillogenin fusion protein expression is stimulated by addition of IPTG. The expressed GST fusion protein is purified by affinity chromatography and separated from its GST fusion partner by cleavage with the protease Factor Xa (Pharmacia). Then, the Factor Xa is removed from the preparation by benzamidine Sepharose 6B beads. Protein purity before injection into rabbits is checked using SDS-PAGE and Coomassie blue staining.

Purified recombinant or extracted oscillogenin is injected into rabbits to produce polyclonal antibodies. The immunization procedure involves an initial injection (40 µg of oscillogenin) followed by two boost injections of 20 µg of protein 3 to 4 weeks apart.

Polyclonal antibodies thus raised can be affinity purified, eluted via a pH gradient, and stored in a borate buffer.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

## Claims

I Claim:

1. A method of isolating an oscillogenin from sperm comprising:

(A) preparing a sperm cytoplasmic fraction;

(B) isolating oscillogenin by sequentially processing the sperm cytoplasmic fraction through a HiTrap blue affinity FPLC chromatographic column, a hydroxyapatite FPLC column, and a Superose 12 FPLC chromatographic column; and

(C) obtaining a fraction with $[Ca^{2+}]_i$ releasing activity.

2. A method of enhancing oocyte activation comprising the step of:

(a) introducing oscillogenin into an oocyte prior to, simultaneously with, or immediately after injecting or fusing the oocyte with a sperm or other cell nuclei, wherein said oocyte has been treated, before or after oscillogenin injection, to remove or inactivate its endogenous nucleus.

3. The method of claim 2, wherein the oocyte is a mammalian oocyte.

4. The method of claim 3, wherein the mammalian oocyte is a human oocyte.

5. The method of claim 2 further comprising incubation of the injected oocyte in a medium containing $Ca^{2+}$.

6. The method of claim 2, wherein the sperm is a mammalian sperm.

7. The method of claim 6, wherein the mammalian sperm is selected from the group consisting of: primate, bovine, porcine, ovine, equine, feline, canine, murine and caprine.

8. The method of claim 2, further comprising the step of injecting the oocyte with at least one agent which additionally enhances divalent cation release or a combination of such agents.

9. The method of claim 8, wherein the agent is selected from the group consisting of: a calcium ionophore, a protein kinase inhibitor and a phosphatase.

10. The method of claim 9, wherein the calcium ionophore is selected from the group consisting of: ionomycin and A23187.

11. The method of claim 9, wherein the protein kinase inhibitor is selected from the group consisting of: 6-dimethylaminopurine (DMAP), staurosporine, butyrolactone, roscovitine, p34(cdc2) inhibitors, 2-aminopurine and sphingosine.

12. The method of claim 9, wherein the phosphatase is select from the group consisting of: phosphatase 2A and phosphatase 2B.

13. The method of claim 2, which further comprises allowing said activated oocyte to develop into an embryo.

14. The method of claim 13, wherein said embryo is non-human, and is implanted into a female surrogate.

15. The method of claim 14, wherein said implanted embryo is allowed to develop into a viable, non-human offspring.

16. The method of claim 2, wherein said activated oocyte is cultured to produce a blastocyst.

17. The method of claim 16, which further comprises culturing all or part of the inner cell mass of said blastocyst on a feeder layer to produce a cultured inner cell mass.

18. The method of claim 17, wherein said cultured inner cell mass is transferred onto a different feeder layer in order to prevent differentiation of said cultured inner cell mass.

19. The method of claim 18, wherein said cultured inner cell mass is cultured to produce a cultured inner mass cell line.

20. A method of enhancing intracytoplasmic sperm injection (ICSI) comprising the step of injecting an oocyte with oscillogenin either before or after a sperm or sperm nucleus is inserted into the oocyte.

21. The method of claim 20 further comprising incubation of the injected oocyte in a medium containing $Ca^{2+}$.

22. The method of claim 20, wherein the oocyte and sperm are mammalian.

23. The method of claim 22, wherein the oocyte is selected from the group consisting of: primate, bovine, porcine, ovine, equine, feline, canine, murine and caprine.

24. The method of claim 22, wherein the sperm is selected from the group consisting of: primate, bovine, porcine, ovine, equine, feline, canine, murine and caprine.

25. The method of claim 20, further comprising the step of injecting the oocyte with at least one agent which enhances divalent cation release.

26. The method of claim 25, wherein the agent is selected from the group consisting of: a calcium ionophore, a protein kinase inhibitor and a phosphatase.

27. The method of claim 26, wherein the calcium ionophore is selected from the group consisting of: ionomycin and A23187.

28. The method of claim 26, wherein the protein kinase inhibitor is selected from the group consisting of: 6-dimethylaminopurine (DMAP), staurosporine, butyrolactone, roscovitine, p34(cdc2) inhibitors, 2-aminopurine and sphingosine.

29. The method of claim 26, wherein the phosphatase is select from the group consisting of: phosphatase 2A and phosphatase 2B.

30. A method of parthenogenically activating an oocyte comprising the step of injecting ooscillogenin into the oocyte.

31. The method of claim 30, wherein the oocyte is a mammalian oocyte.

32. The method of claim 31, wherein the mammalian oocyte is selected from the group of mammals consisting of: human, primate, bovine, porcine, ovine, equine, feline, canine, murine and caprine.

33. A method of predicting sperm $[Ca^{2+}]_i$ releasing activity comprising measuring oscillogenin concentration in a sperm sample.

34. A kit for predicting sperm $[Ca^{2+}]_i$ releasing activity comprising a labeled agent which recognizes and binds to oscillogenin or a nucleic acid encoding oscillogenin.

35. The kit of claim 34, wherein the agent is an anti-oscillogenin antibody.

36. The kit of claim 31, wherein the agent is a nucleic acid probe which binds to oscillogenin mRNA.

37. A nucleic acid encoding an oscillogenin.

38. The nucleic acid of claim 37, wherein the nucleic acid comprises SEQ ID NO. 1.

39. A vector comprising the nucleic acid of claim 37.

40. The nucleic acid of claim 37, wherein the oscillogenin is a mammalian oscillogenin.

41. The nucleic acid of claim 40, wherein the mammalian oscillogenin is selected from the listing consisting of human, bovine, porcine, ovine, equine, feline, canine, murine and caprine.

42. An oscillogenin protein encoded by the nucleic acid of claim 38.

43. An oscillogenin protein comprising SEQ ID NO: 2.

44. An oscillogenin protein comprising at least twenty (20) consecutive amino acid residues of SEQ ID NO.: 2.

45. An isolated oscillogenin obtained by the method of claim 1.

46. A recombinant oscillogenin protein obtained by:

(A) inserting the vector of claim 39 into a suitable host;

(B) incubating said host under suitable conditions to produce oscillogenin; and

(C) isolating oscillogenin protein from said host.

47. A composition for activating oocytes comprising an oscillogenin protein and a pharmaceutically acceptable carrier.

48. The composition of claim 46 further comprising at least a phosphatase, a calcium ionophore or a protein kinase inhibitor.

49. An antibody or immunogenic fragment thereof which recognizes and binds to oscillogenin.

50. The antibody of claim 48, wherein the antibody is a monoclonal antibody.

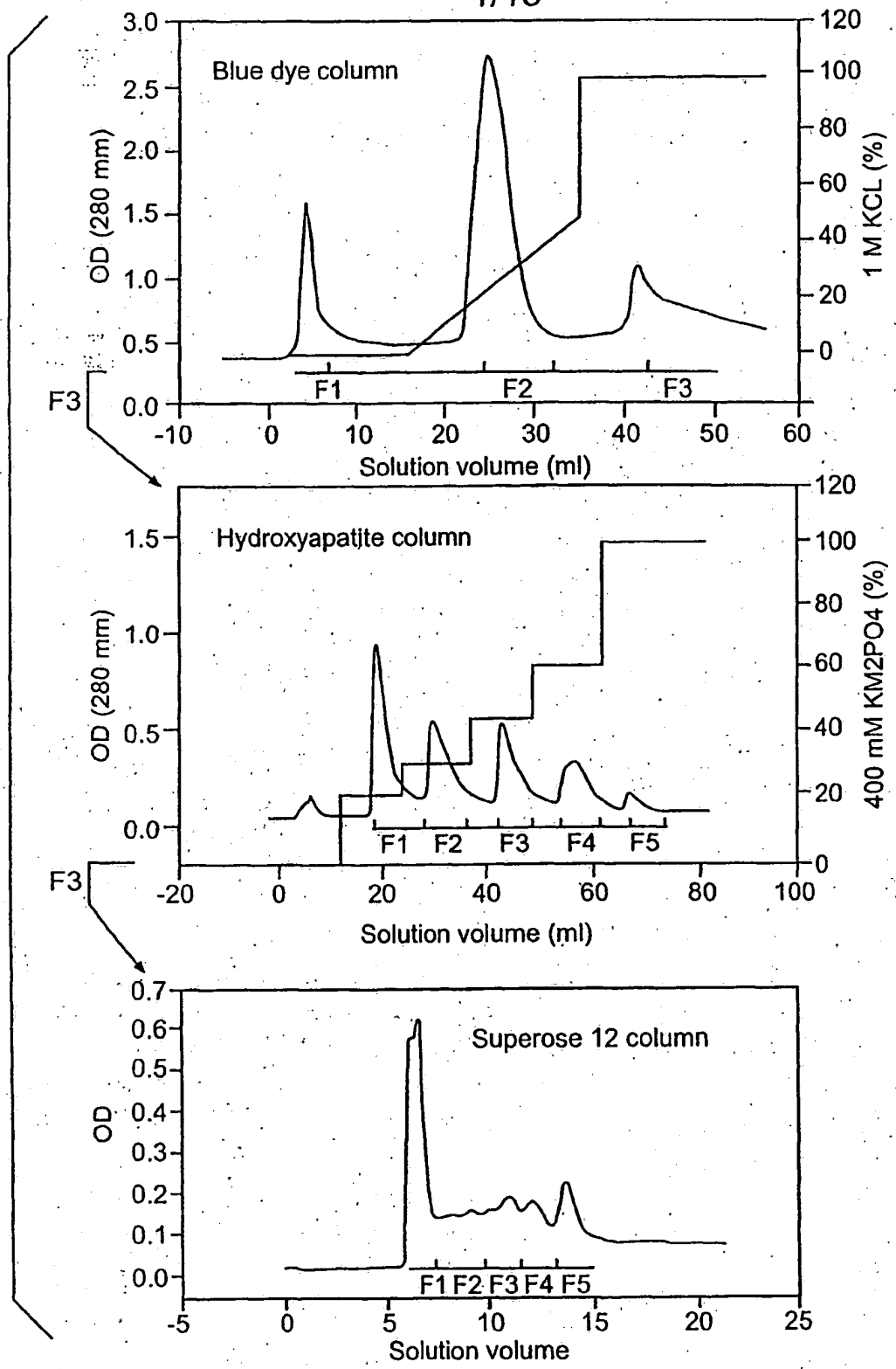
51. The antibody or immunogenic fragment of claim 48, wherein the immunogenic fragment is selected from the group consisting of: Fab, scFv, $F(ab')_2$ and Fab'.

52. The antibody or immunogenic fragment of claim 48, wherein the antibody or immunogenic fragment is a labeled antibody.

53. The antibody or immunogenic fragment of claim 51, wherein the antibody or immunogenic fragment is labeled with an isotope or a fluorescent label.

54. The antibody of claim 52, wherein the fluorescent label is rhodamine, fluorescein or Rhodamine GreenÔ.

55. A method for inhibiting sperm fertility comprising the step of administering an agent which inhibits oscillogenin activity in sperm.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OD (280 mm)
1 M KCL (%)
F1
F2
F3
Solution volume (ml)
Hydroxyapatite column
OD (280 mm)
400 mM KM2PO4 (%)
F1 F2 F3 F4 F5
Solution volume (ml)
Superose 12 column
OD
F1 F2 F3 F4 F5
Solution volume

FIG. 1A

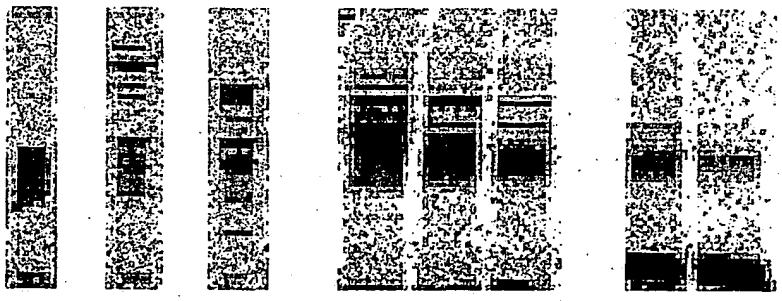

FIG. 1B

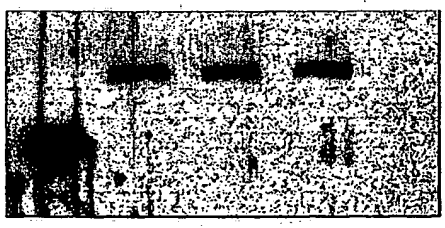

FIG. 2A

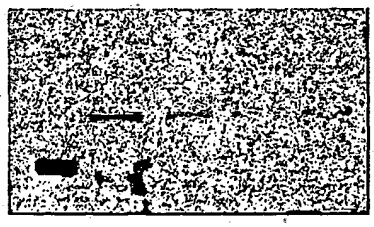

FIG. 2C

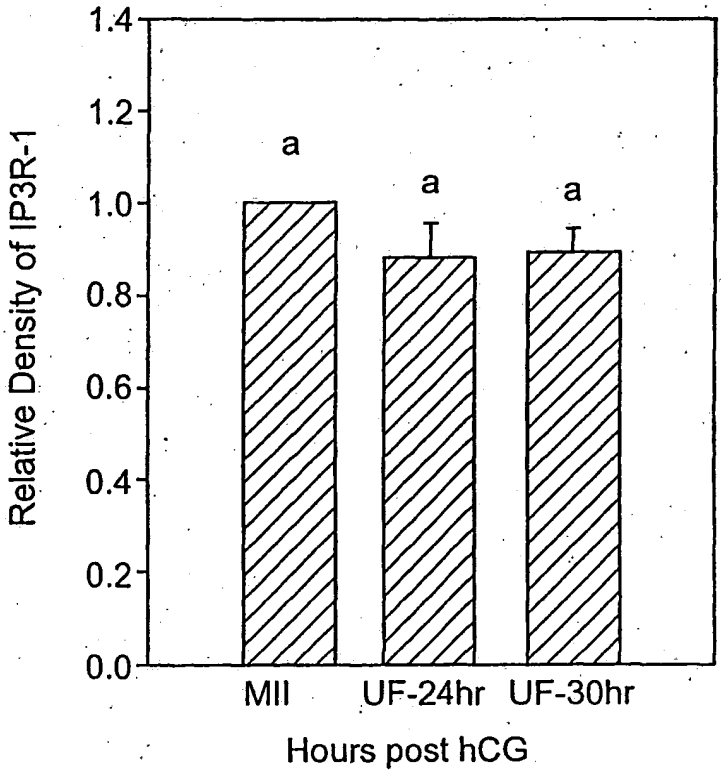
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Density of IP3R-1
a
a
a
MII
UF-24hr
UF-30hr
Hours post hCG

FIG. 2B

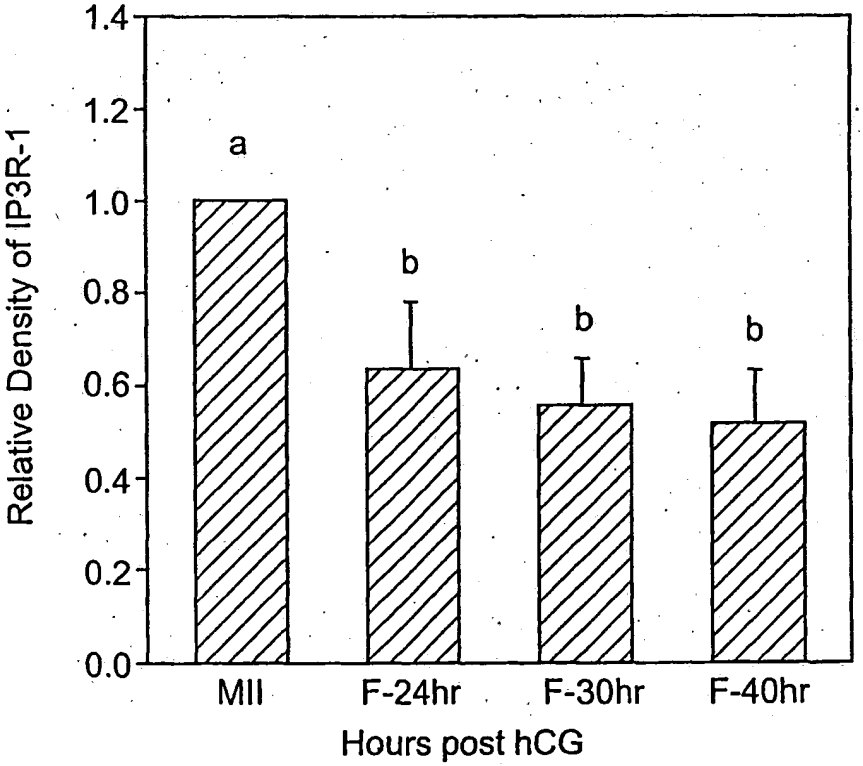
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Density of IP3R-1
a
b
b
b
MII
F-24hr
F-30hr
F-40hr
Hours post hCG

FIG. 2D

FIG. 3A

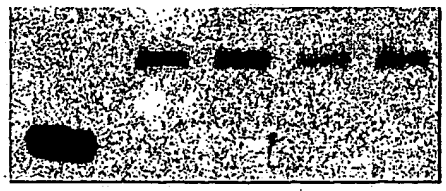

FIG. 3C

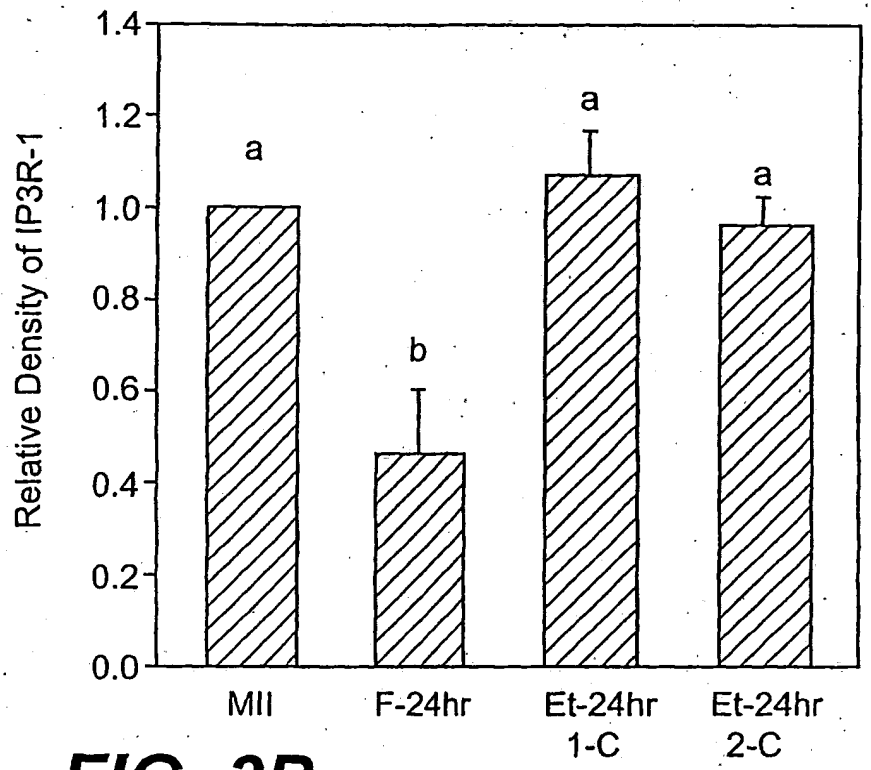
Relative Density of IP3R-1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
a
MII
F-24hr
Et-24hr 1-C
Et-24hr 2-C
Hours post hCG

FIG. 3B

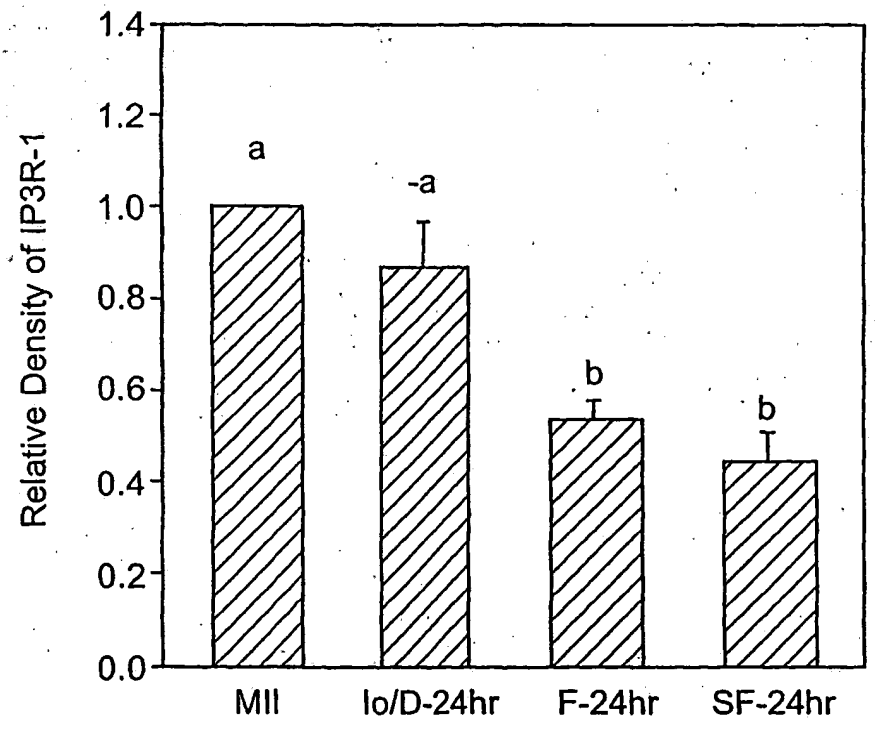
Relative Density of IP3R-1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
-a
b
b
MII
Io/D-24hr
F-24hr
SF-24hr
Hours post hCG

FIG. 3D

FIG. 4A

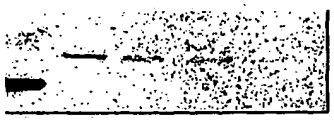

FIG. 4C

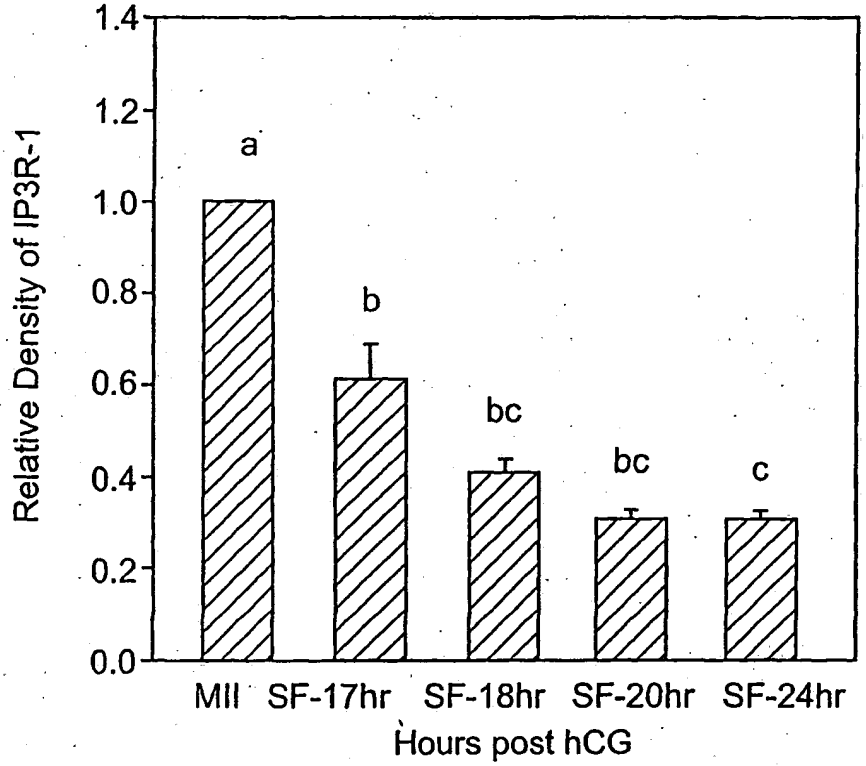
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Density of IP3R-1
a
b
bc
bc
c
MII
SF-17hr
SF-18hr
SF-20hr
SF-24hr
Hours post hCG

FIG. 4B

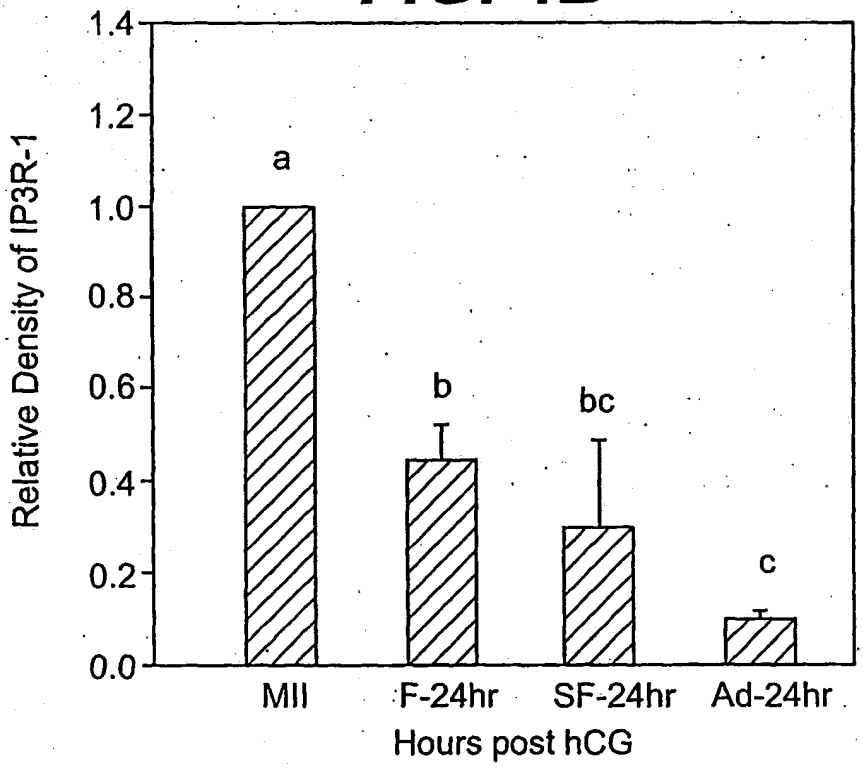
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Density of IP3R-1
a
b
bc
c
MII
F-24hr
SF-24hr
Ad-24hr
Hours post hCG

FIG. 4D

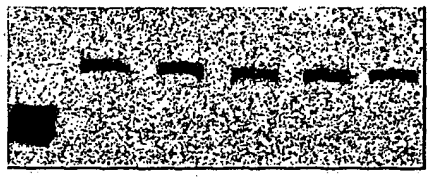

FIG. 5A

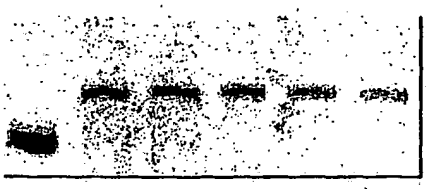

FIG. 5C

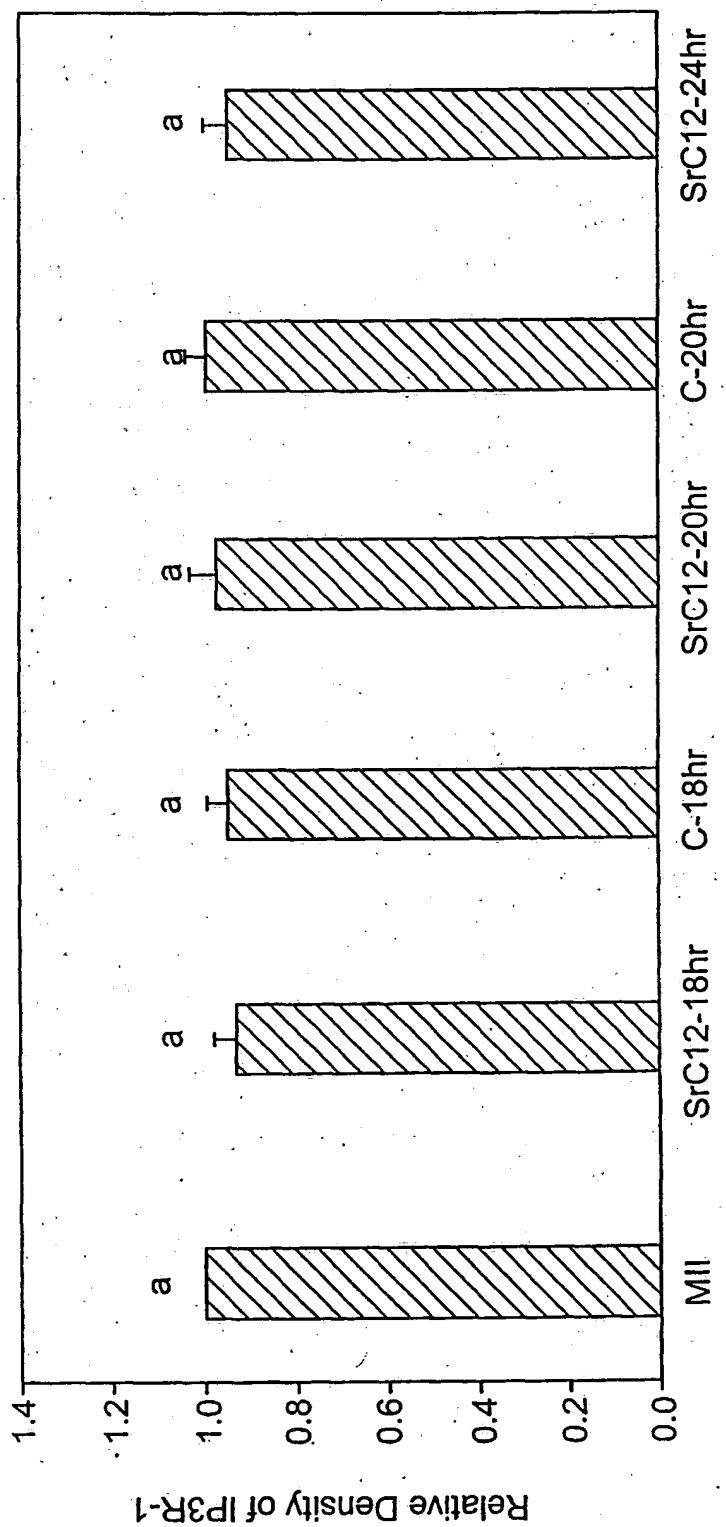
Relative Density of IP3R-1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
a
a
a
MII
SrC12-18hr
C-18hr
SrC12-20hr
C-20hr
SrC12-24hr
Hours post hCG

FIG. 5B

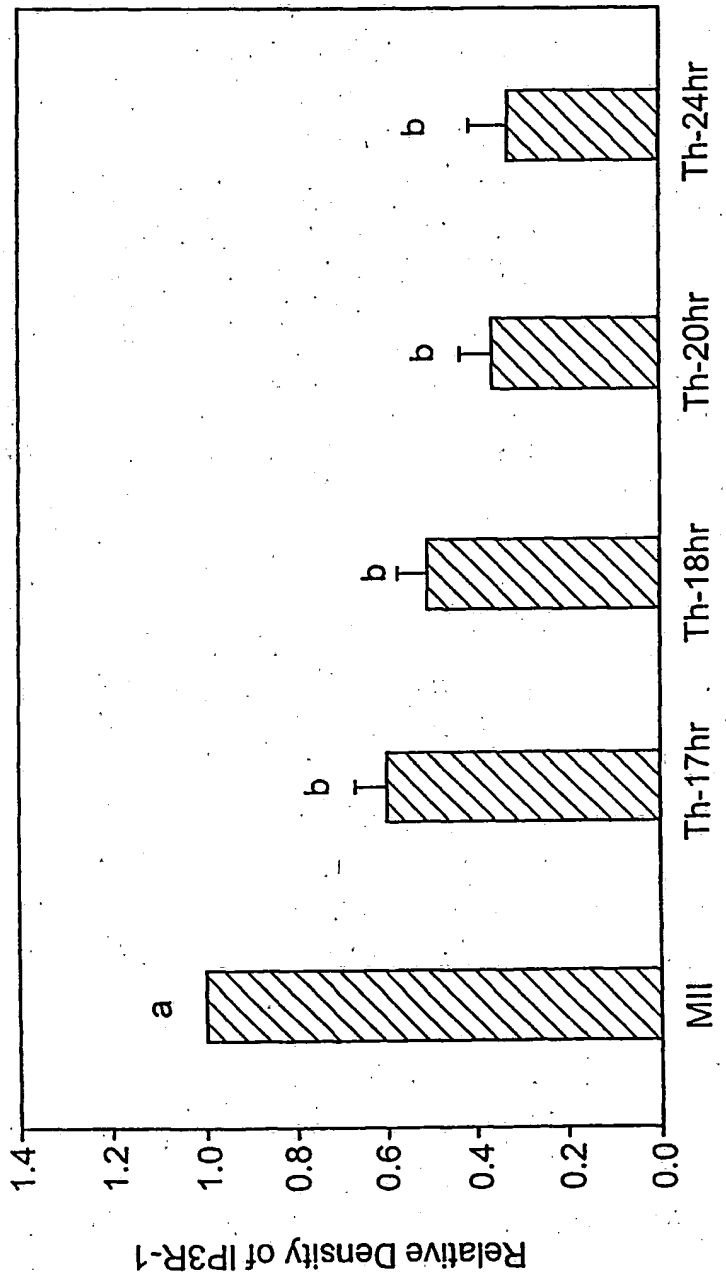
Relative Density of IP3R-1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
b
b
MII
Th-17hr
Th-18hr
Th-20hr
Th-24hr
Hours post hCG

FIG. 5D

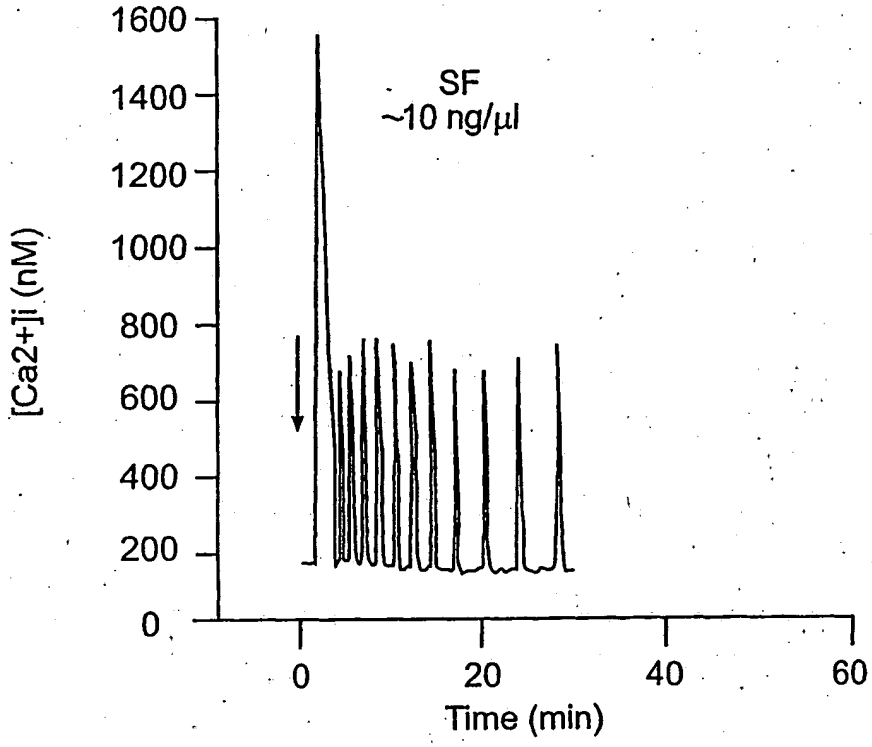
1600
1400
1200
1000
800
600
400
200
0
[Ca2+]i (nM)
SF
~10 ng/µl
0
20
40
60
Time (min)

FIG. 6A

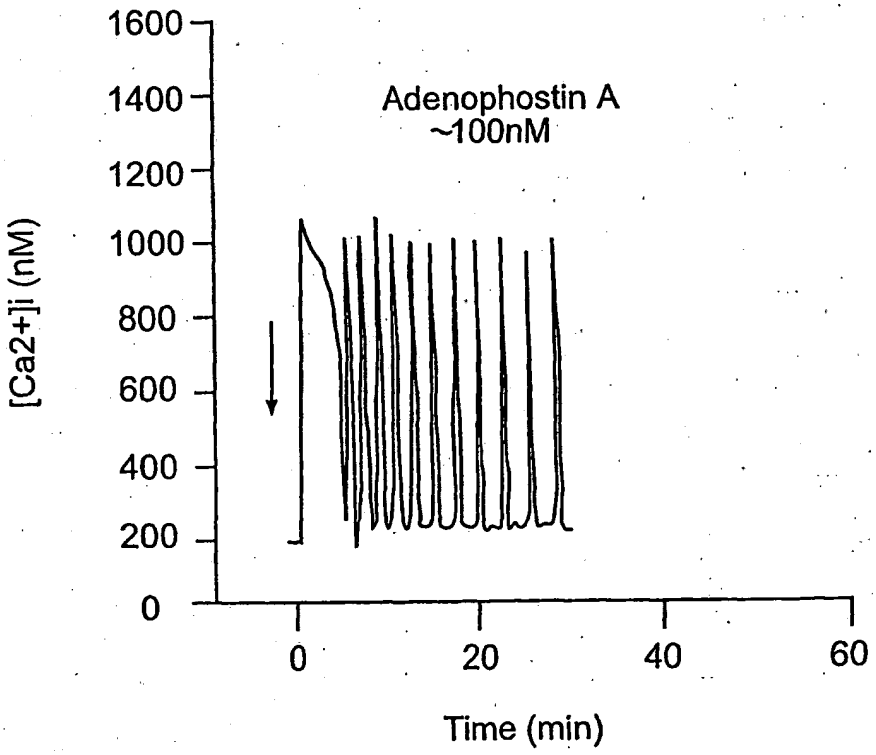
1600
1400
1200
1000
800
600
400
200
0
[Ca2+]i (nM)
Adenophostin A
~100nM
0
20
40
60
Time (min)

FIG. 6B

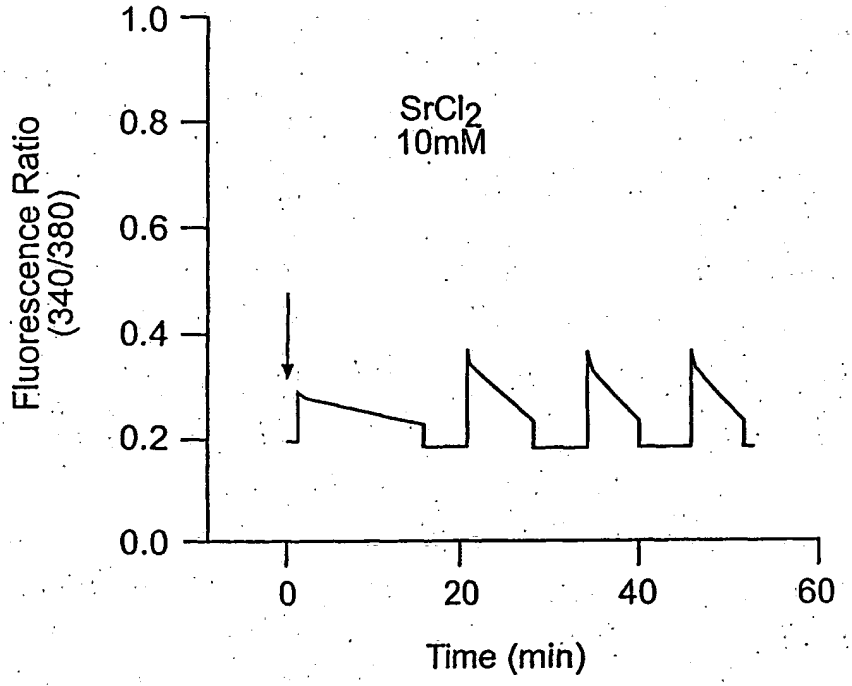
1.0
0.8
0.6
0.4
0.2
0.0
Fluorescence Ratio (340/380)
SrCl2 10mM
0
20
40
60
Time (min)

FIG. 6C

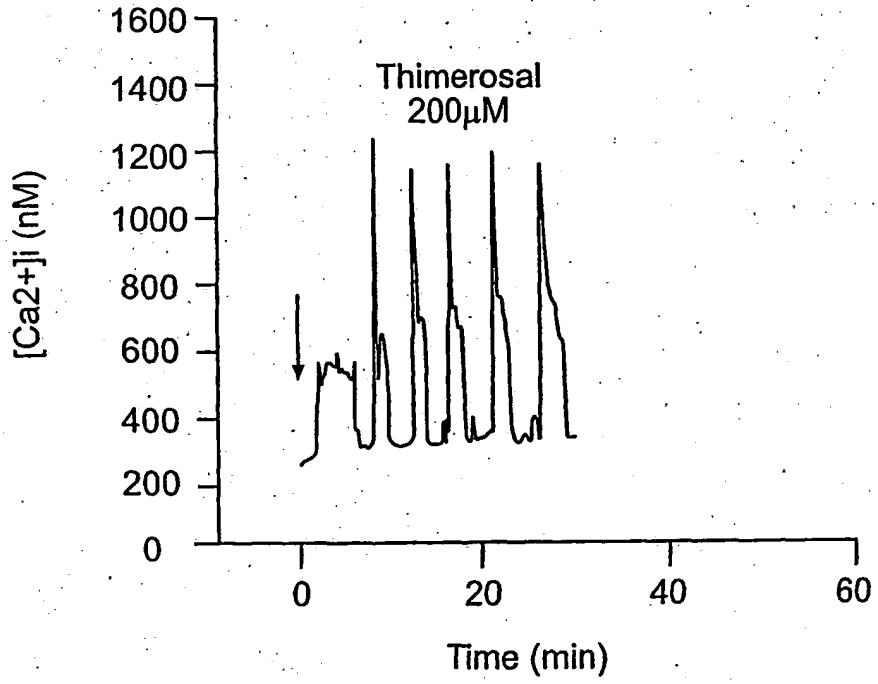
1600
1400
1200
1000
800
600
400
200
0
[Ca2+]i (nM)
Thimerosal 200µM
0
20
40
60
Time (min)

FIG. 6D

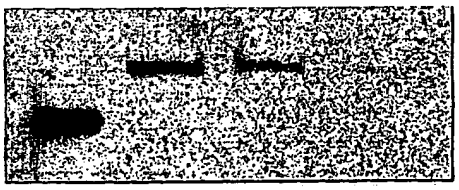

FIG. 7A

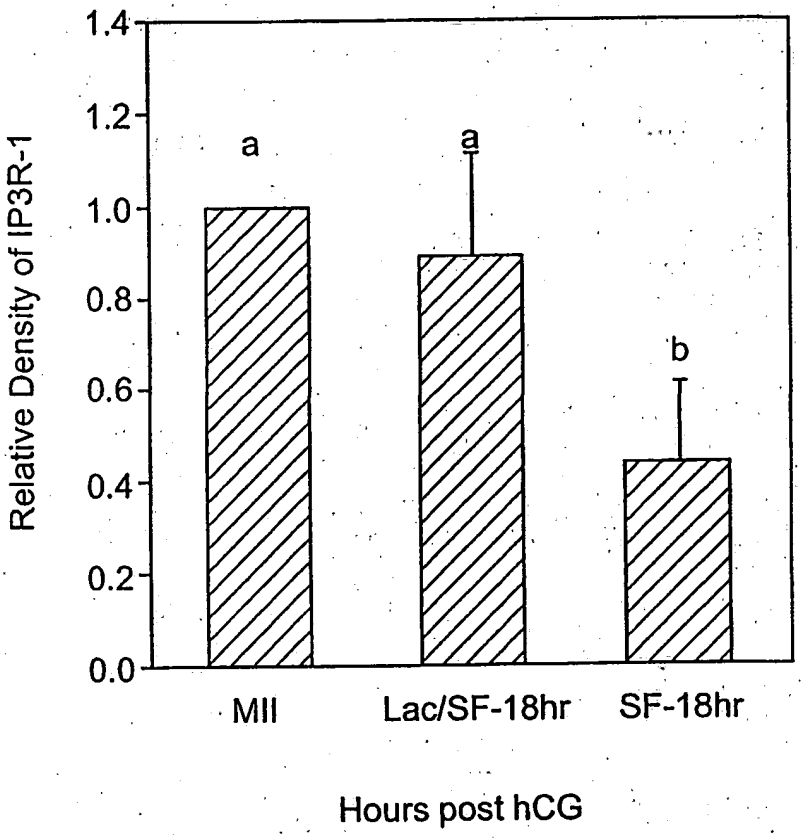
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Density of IP3R-1
a
a
b
MII
Lac/SF-18hr
SF-18hr
Hours post hCG

FIG. 7B

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL : 424/811, 198.1; 435/4,69.1; 530/389.2, 413, 852; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/811, 198.1; 435/4,69.1; 530/389.2, 413, 852; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG MEDLINE EMASE BIOSIS LIFESCI CHEM ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WITTON et al. Injection of a boar sperm factor causes calcium oscillations in oocytes of the marsupial opossum, Monodelphis domestica. Zygote. November 1999, Vol. 7, No. 4, pages 271-277, see entire document. | 1-37, 39-42, 44-55 |
| Y,P | PARRINGTON et al. The soluble mammalian sperm factor protein that triggers $Ca^{2+}$ oscillations in eggs: Evidence for expression of mRNA(s) coding for sperm factor protein(s) in spermatogenic cells. Biology of the Cell. 2000, Vol. 92, No. 3/4, pages 267-275, see entire document. | 1-37, 39-42, 45-55 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>28 JULY 2001 | Date of mailing of the international search report<br>07 AUG 2001 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Patrick Nolan<br>Telephone No. (703) 38-0196 |

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y,P | TANG et al. $Ca^{2+}$ oscillations induced by a cytosolic sperm protein factor are mediated by a material machinery that functions only once in mammalian eggs. Development. 2000, Vol. 127, No. 5, pages 111-1150, see entire document. | 1-37, 39-42, 45-55 |
| Y,P | GORDO et al. *Injection of sperm cytosolic factor into mouse metaphase II oocytes induces different developmental fates according to the frequency of* $[Ca^{2+}]_i$ oscillations and oocyte age. Biology of Reproduction. May 2000, Vol. 62, No. 5, pages 1370-1379, see entire document. | 1-37, 39-42, 45-55 |



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 39/00; A23J 1/00; C07H 21/04; C07K 16/00; C12P 21/08; C12Q 1/00

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 38, 43, 44
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims have SEQ ID NOS. but no CRF was filed with the case

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL : 424/811, 198.1; 435/4,69.1; 530/389.2, 413, 852; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/811, 198.1; 435/4,69.1; 530/389.2, 413, 852; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG MEDLINE EMASE BIOSIS LIFESCI CHEM ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WITTON et al. Injection of a boar sperm factor causes calcium oscillations in oocytes of the marsupial opossum, Monodelphis domestica. Zygote. November 1999, Vol. 7, No. 4, pages 271-277, see entire document. | 1-37, 39-42, 44-55 |
| Y,P | PARRINGTON et al. The soluble mammalian sperm factor protein that triggers $Ca^{2+}$ oscillations in eggs: Evidence for expression of mRNA(s) coding for sperm factor protein(s) in spermatogenic cells. Biology of the Cell. 2000, Vol. 92, No. 3/4, pages 267-275, see entire document. | 1-37, 39-42, 45-55 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>28 JULY 2001 | Date of mailing of the international search report<br>07 AUG2001 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Patrick Nolan<br>Telephone No. (703) 38-0196 |

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y,P | TANG et al. $Ca^{2+}$ oscillations induced by a cytosolic sperm protein factor are mediated by a material machinery that functions only once in mammalian eggs. Development. 2000, Vol. 127, No. 5, pages 111-1150, see entire document. | 1-37, 39-42, 45-55 |
| Y,P | GORDO et al. *Injection of sperm cytosolic factor into mouse metaphase II oocytes induces different developmental fates according to the frequency of* $[Ca^{2+}]_i$ *oscillations and oocyte age.* Biology of Reproduction. *May 2000, Vol. 62, No. 5, pages 1370-1379, see entire document.* | 1-37, 39-42, 45-55 |

Form PCT/ISA/210 (continuation of second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 39/00; A23J 1/00; C07H 21/04; C07K 16/00; C12P 21/06; C12Q 1/00

Form PCT/ISA/210 (extra sheet) (July 1998)*

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/08998

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 38, 43, 44
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims have SEQ ID NOS. but no CRF was filed with the case

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.



SUBSTITUTE SHEET (RULE 26)